CHEMICAL CONSTITUENTS OF *OCHREINAUCLEA MAINGAYII* (HOOK.F.) RIDSD. (RUBIACEAE)

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FACULTY OF SCIENCE

UNIVERSITY OF MALAYA

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF SCIENCE

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ABSTRACT

A study of chemical constituents from the leaves and bark of *Ochreinauclea maingayii* (Hook. f.) Ridsd. from the family Rubiaceae has been carried out. Nine compounds were successfully isolated and purified by applying chromatography techniques such as column chromatography (CC), thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC). The compounds were harmane **38**, naucledine **5**, cadamine **42**, isodihydrocadambine **78**, neonaucline **79**, cinnamide **81**, benzamide **80**, blumenol A **75** and ursolic acid **73**. Neonaucline **75** is a new compound from the leaves of this species. All structures were elucidated by the means of spectroscopic method; ^{1}H, ^{13}C NMR, 2D NMR (COSY, HMBC, HMQC, NOESY and DEPT), UV, IR and LCMS. The significant vasorelaxant activity (more than 80% relaxation) could be observed on rat aorta was observed for compound naucledine **5**, cadamine **42** and neonaucline **79** after injection of each sample with 1×10^{-5} M.

ABSTRAK

Kajian sebatian kimia daripada bahagian daun dan batang spesies *Ochreinauclea maingayii* (Hook. f.) Ridsd. dari keluarga Rubiaceae telah dijalankan. Sembilan sebatian telah berjaya dipisahkan dan ditulenkan menggunakan kaedah kromatografi seperti kromatografi turus, kromatografi lapisan nipis dan juga lapisan nipis persediaan. Sebatian-sebatian adalah harman **38**, naukledina **5**, kadamina **42**, isodihidrokadambina **78**, neonauklina **79**, cinnamida **81**, benzamida **80**, blumenol A **75** dan asid ursolik **73**. Neonauklina **77** adalah sebatian baru daripada bahagian daun spesies ini. Semua struktur sebatian tulen yang diperolehi ditentukan melalui kaedah spektroskopi ^{1}H,^{13}C NMR, 2D-NMR (COSY, HMBC, HMQC, NOESY dan DEPT), UV, IR and LCMS. Aktiviti pengenduran-vaso yang sangat baik (lebih dari 80% kerehatan) pada aorta tikus dapat diperhatikan untuk. sebatian naukledina **5**, kadamina **42** dan neonauklina **79** selepas disuntik pada kepekatan 1×10^{-5} M bagi setiap sampel .

ACKNOWLEDGEMENT

Firstly, I want to thank to Almighty Allah S.W.T, Most Gracious and Most Merciful. Here, I would like to thank my supervisor, Prof. Dr. Khalijah Awang for her invaluable time spent in providing me the guide, suggest, concern and courage throughout for completion of my thesis.

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CONTENTS

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

α	Alfa
β	Beta
δ	Chemical shift
Kg	Kilogram
ppm	Parts per million
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
ml	Milliliter
M	Molar
m	Meter
µM	Micromolar
MHz	Mega Hertz
Hz	Hertz
UV	Ultraviolet
λ	Maximum wavelength
IR	Infrared
cm^{-1}	Per centimetre
J	Coupling constant (Hz)

s	Singlet
t	Triplet
d	Doublet
dd	Doublet of doublet
m	Multiplet
NMR	Nuclear Magnetic Resonance
^{1}H	Proton
^{13}C	13-Carbon
COSY	^{1}H-^{1}H Correlations Spectroscopy
$CDCl_3$	Deutrated Chloroform
CH_3	Methyl Group
OH	Hydroxyl Group
MS	Mass Spectrum
m/z	Mass/Charge
TLC	Thin Layer Lhromatography
PTLC	Preparative Thin Layer Chromatography
CC	Column Chromatography
MC	Micro Column Chromatography
HCl	Hydrochloric Acid
eV	Electron Volt

Na_2SO_4	Sodium Sulphate
H_2SO_4	Sulphuric Acid
$MgSO_4$	Magnesium Sulphate
NaCl	Sodium Chloride
KCI	Potassium Chloride
°C	Degree Celsius
2D NMR	Two Dimensional NMR
DEPT	Distortion Less Enhancement by Polarisation Transfer
GCMS	Gas Chromatogram Mass Spectroscopy
LCMS-IT-TOFF	Liquid Chromatogram Mass Spectroscopy Ion Trap Time of Flight
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.0 General

Natural products have been investigated and utilized to alleviate disease since early human history, and today the natural substances have served as one of the most significant source of new leads for pharmaceutical development.[1] Many compounds which occur in nature possess properties which are attractive to people such as perfumes, medicinal portion, dye and poison. The application of modern experimental techniques has facilitated the isolation of pure compounds from plant preparation and thus enables the chemist and biochemist to examine in detail the structure and chemistry of the compound responsible effects. The isolation of natural products that have biological activities against organism has some advantages, including pure active compound can be synthesized to produce more compounds, to develop analytic assay for a particular compound and it permits the structural determination of bioactive compounds.

The tropical rain forest in Malaysia comprises more than 15,000 flowering plants and 1170 ferns species, many of them have been claimed to possess some medicinal properties or biological activities.[2] Among the plants possessing such interesting activities are from families of Leguminoceae, Rubiaceae, Lauraceae, Apocynaceae, Rutaceae, Moraceae, Meliaceae, and Anonaceae.[3] Regarding research and development on Malaysian medicinal plants, there remain a dire need for well-coordinated research program involving botanist, pharmacologist and microbiologist, resulting in the publication and dissemination of basic and applied scientific information, essential for the development and commercialization of health products including medicines and nutraceuticals.[4]

Thus, in view of the important of drug discovery and the abundant resource, the author has embarked a study of the chemical constituents of the *Ochreinauclea maingayii*. From the literatures study, the author has found that the genus Ochreinauclea from Rubiaceae has not been the subject of any chemical and biological studies. Therefore, in the effort to discover new active molecules, the study mainly deals with isolation, structure elucidation of natural products from the bark and leaves.

The objectives of this study are as follows:

1. To isolate the chemical constituents from the bark and leaves of *Ochreinauclea maingayii*.
2. To elucidate the structure of both unknown and known compounds from *Ochreinauclea maingayii*. The compounds will be analyzed using spectroscopic methods mainly 2D NMR (COSY, HMBC, HMQC and NOESY), UV and IR.
3. To investigate the bioactivity of the crude and major compounds toward vasorelaxant activity.

1.1 Rubiaceae: Distribution and Habitat

Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs, less often of perennial to annual herbs, as in Rubiaceae (subfamily Rubioideae) which are found in temperate regions. The Rubiaceae family is one of the largest of the angiosperms, with 10,700 species distributed in 637 genera.[5] It is the biggest family of flowering plants (after Orchidaceae, Compositae, Leguminosae, Gramineae). In Malaya, it is the biggest family of trees, represented by 80 genera and 555 species. Four genera (*Aleisanthia, Klossia, Kochummenia, Perakanthus*) are endemic to Malaya.[6] The most recent and complete classification subdivided this large family into four subfamilies, namely Cinchonoideae, Ixoroideae, Antirheoideae and Rubioideae, corresponding to about 50 tribes.[5]

The Rubiaceae are an important component of the lower strata of the rain forest. Only about a dozen species grow to 30 m or taller and reaching 2 m in girth, the commonest being *Jackiopsis ornata, Metadina trichotoma, Mussaendopsis beccariana, Nauclea officinalis, Neolamarckia cadamba* and *Rothmannia schoemannii.*[6]

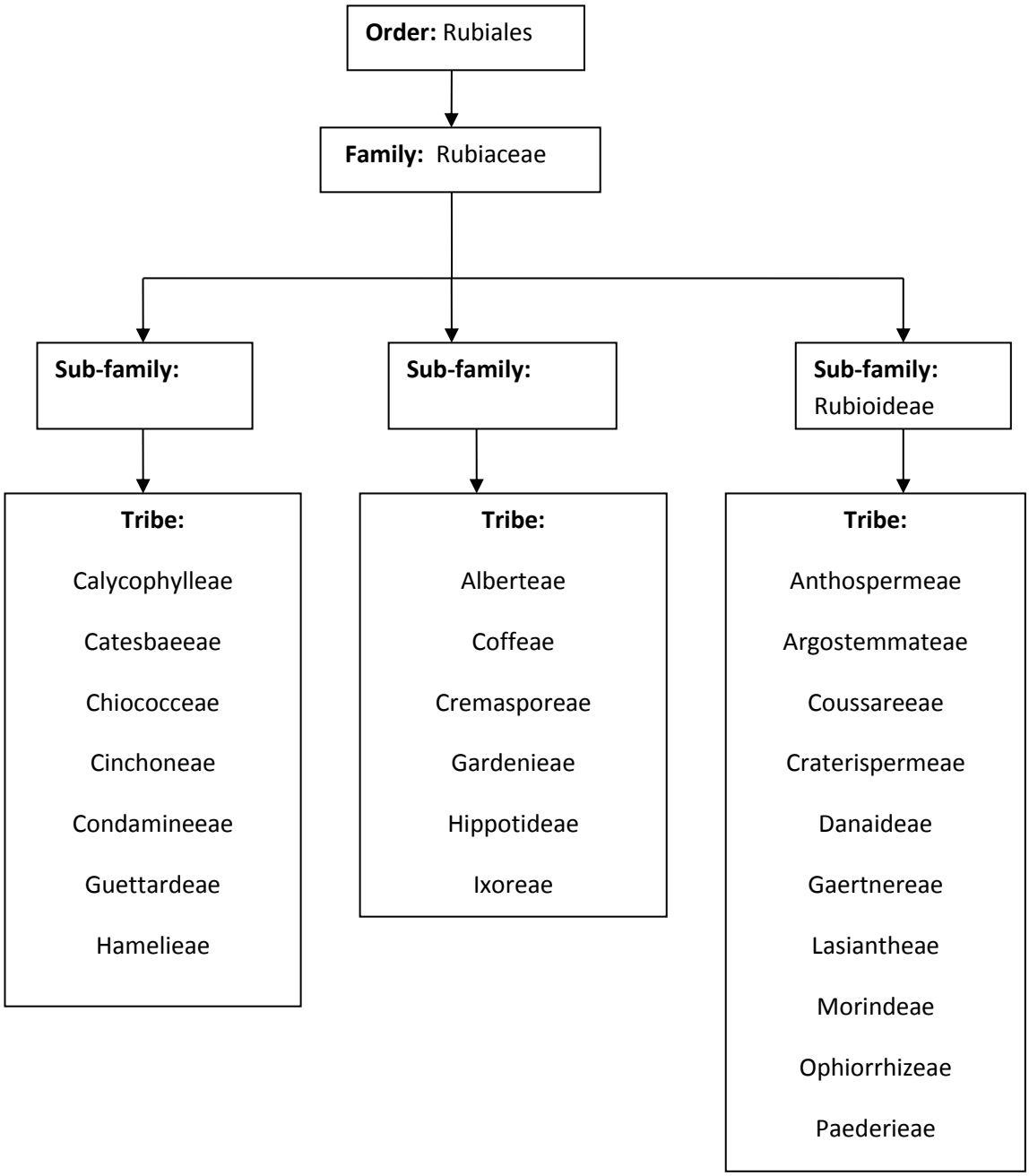
In Malaysia the only timbers of note in the family of Rubiaceae are "bangkal" (*Nauclea, Neonaucleae, Orchreinauclea*), "laran" (*Neolamarckia cadamba*), "malabera bukit" (*Mussaendopsis beccariana*), "mengkudu" (*Morinda*), "meraga" (*Metadina, Pertusadina*), "selumar" (*Jackiopsis ornata*) and "tinjau belukar" (*Porterandia anisophylla*). The best known economic products of the family Rubiaceae are coffee, gambier, quinine and ipecacuanha.[6] Rubiaceae is also famous with its trade name 'Kopi'.

1.2 General Appearance and Morphology of Rubiaceae Family

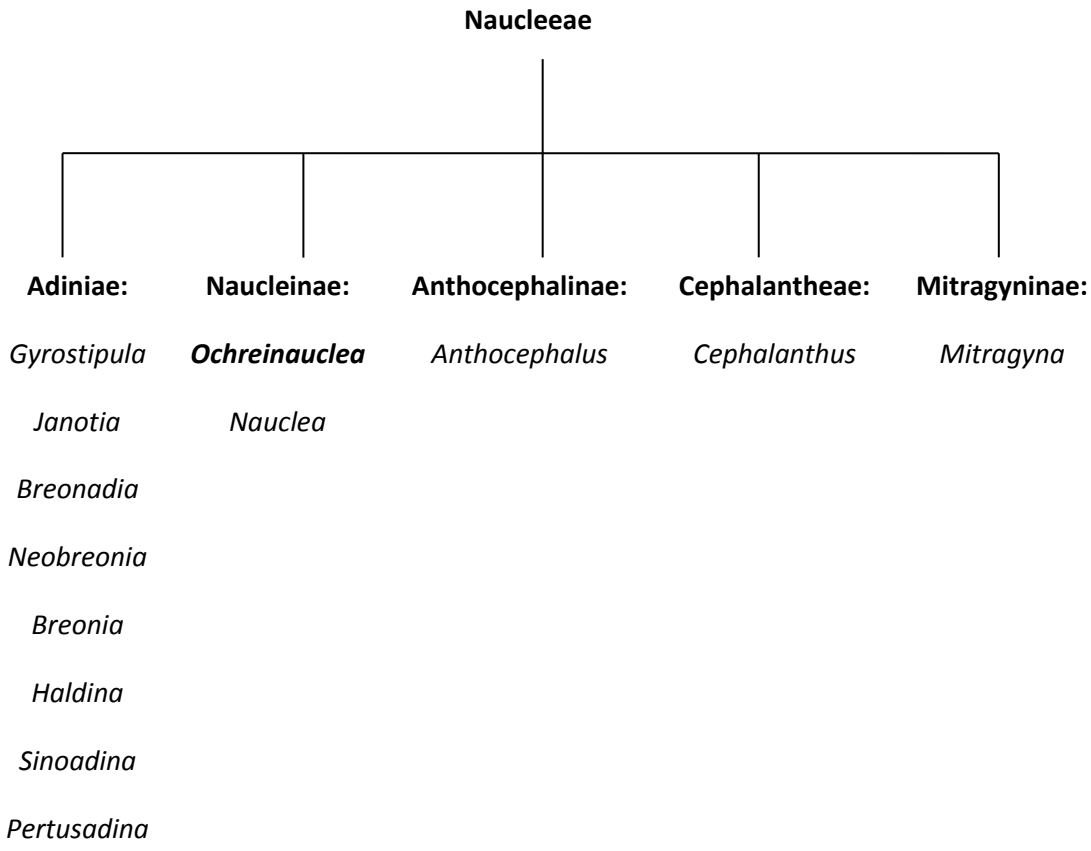
Rubiaceae are herbs or shrubs, terrestrial, epiphytic, creeping, climbing or scrambling trees, rarely (*Aidiopsis*) stranglers. It can be recognized by its stems or trunks which are unarmed or spiny with a swollen base containing ant-inhabited channels. Inner bark without milky latex, sometimes yielding a clear yellow sap when cut (e.g., *Morinda, Rothmannia*). Leaves opposite, in two rows or decussate or in whorls, in some taxa with one leaf reduced at successive nodes or alternate nodes. Flowers bisexual or unisexual, mostly 4-5 merous calyces free or initially apically fused and later free (*Ochreinauclea*) or persistently fused (*Morinda, Nauclea and Rennellia*), sometimes with one lobe enlarged and conspicuously coloured (*Mussaenda, Mussaendopsis*).[6]

1.3 Classification of Tribes: Naucleae

The classification of Rubiaceae family proposed by Hsuan Keng can be illustrated in Scheme 1.1.[7] Rubiaceae family is from Order Rubiales and classified to three sub-families such as Cinchonoideae, Ixoroideae and Rubioideae. This classification is also supported by Razafimandimbison et.al.[8] Hsuan Keng classified that nearly 400 genera, chiefly tropical and subtropical; and nearly seventy genera are native to Malaya. The plant investigated in this study is *Ochreinauclea maingayii*, which belongs to the tribe Naucleeae (Scheme 1.2) from the sub-family Cinchonoideae and the genus *Ochreinauclea*.[7]



Scheme 1.1: Classification of tribe of Rubiaceae family



Scheme 1.2: Relationship between the genera of Naucleeae

1.4 The Genus: *Ochreinauclea*

The genus *Ochreinauclea* is a medium size to large tree with cone tips and pyramidal buds. It has opposite leaves or sometimes in whorls of 3 with petiolate, blade charteceous, narrowly triangular and semi persistant. Inflorensense terminal consisting of the solitary flowering head, with numerous, subsessil 5-merous flowers, their ovaries mutually connate at the apices; interfloral bracts and hairs absent. They have two celled ovary, each locule with numerous ovules which placenta attached to the middle of the septum. The fruit partially, cohering, crowned by persistent calyx lobes, eventually breaking apart into semi cocci. The seeds are numerous small, bilaterally compressed,

shortly winged on both sides. From this genus only two have been classified and they are *Ochreinauclea maingayii* and *Ochreinauclea missionis.*[9-11]

1.5 *Ochreinauclea maingayii*

It exists as medium sized trees with about 18m tall and 20cm diameter. The bark is reddish-brown, lenticellate and smooth. The inner bark (Figure 1.2) appears to be pale pinkish yellow. The leaves (Figure 1.1) are opposite or in whorls of 3-4, corraceous and from broadly elliptic to obovate. The apex shortly pointed or cuspidate, base obtuse, and with size 14.5-21.5 cm x 9-15 cm. The colour of the leaves are dark shinning green above, pale glaucous below, midrib sunken above, secondary nerves 13-18 pairs, sunken above, raised below; tertiary nerves and reticulations.[12]

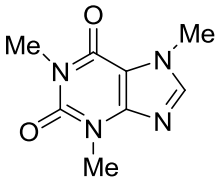
Figure 1.1: Leaves of *Ochreinauclea maingayii*

Figure 1.2: Bark of *Ochreinauclea maingayii*

1.6 Medicinal Values

Rubiaceae are among plants of wide usage in traditional medicines that are continuously screened in laboratory for their pharmacological properties. According to their wide distribution, plants of Rubiaceae are used in all parts of the world as ornamentals, food and remedies.[13] Parts of Rubiaceae plants such as leaves, barks, roots and fruits are used for medical preparations. *Coffea canephora* and *Coffea arabi*ca are two species with most economically important members of the family that are used in the production of coffee. *Coffea arabica* has been known to produce caffeine **1** for stimulant and in the treatment of headache. In medicine, trees of the genus *Cinchona* are of great interest because of their alkaloids, the most familiar being quinine **2**, the first effective agent in treating malaria. Another important drug from the bark of *Cinchona succirubra* is quinidine **3** which is also used as antimalarial agent. Emetine **4** is traditional used as an ametic drug from root of *Cephaelis acuminata* and as a second choice in the treatment of severe intestinal amoebiasis and hepatic amoebiasis when

nitroimidazoles are not effective or contraindicated .[14] More medicinal values from plants of Rubiaceae are listed on Table 1.1.



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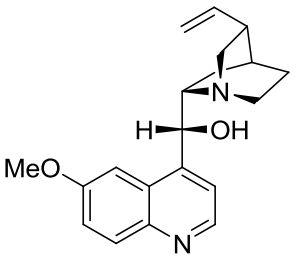

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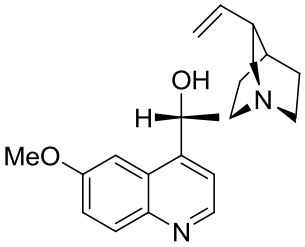

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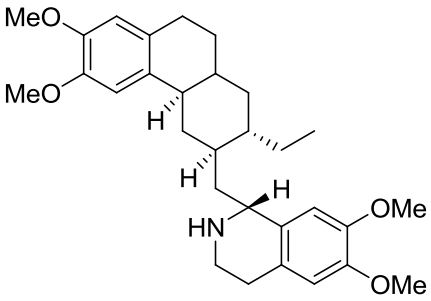

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Table 1.1: Medicinal values of selected plants from Rubiaceae.[15, 16]

Medicinal plants of Rubiaceae Family	Medicinal values
Morinda citrifolia	The leaves are used to treat cough, nausea and colic also to treat gout, tuberculosis and ring worm.
Mitragyna speciosa	The leaves has been reported for its antitussive, anaesthetic, antinociceptive, stimulant, analgesic and narcotic-like actions properties
Dentella repens	The leaves are used in case of blood ailments to purify the blood.
Anthocephalus chinensis	Bark of this plant is used as tonic, febrifuse, antidiuretic and Astringent.
Haldina cordifolia	Bark of this plant is used as febrifuge, antiseptic and aphrodisiac.
Gardenia jasminoides	Root of this plant is used as purgative, an effective cure for indigestion and nervous disorders.
Ixora arborea	Root and fruits are used by tribals to cure micturation and urinary problems of females. Root bark is effective in skin diseases and chest pain.
Hedyotis verticillata	Paste of flower is applied to skin diseases like athlete's foot.
Rubia cordifolia	The whole plant is used in diabetic treatment.
Oldenlandia umbellate	Root and leaves are expectorant and given bronchial disorders.
Mitragyna parviflora	The bark of this plant is prescribed in cases of colic pain and problems like peptic ulcers.
Ixora coccinea	Roots and flowers are used as curative for dysentery and ulcer.

CHAPTER 2

GENERAL CHEMICAL ASPECT

GENERAL CHEMICAL ASPECT

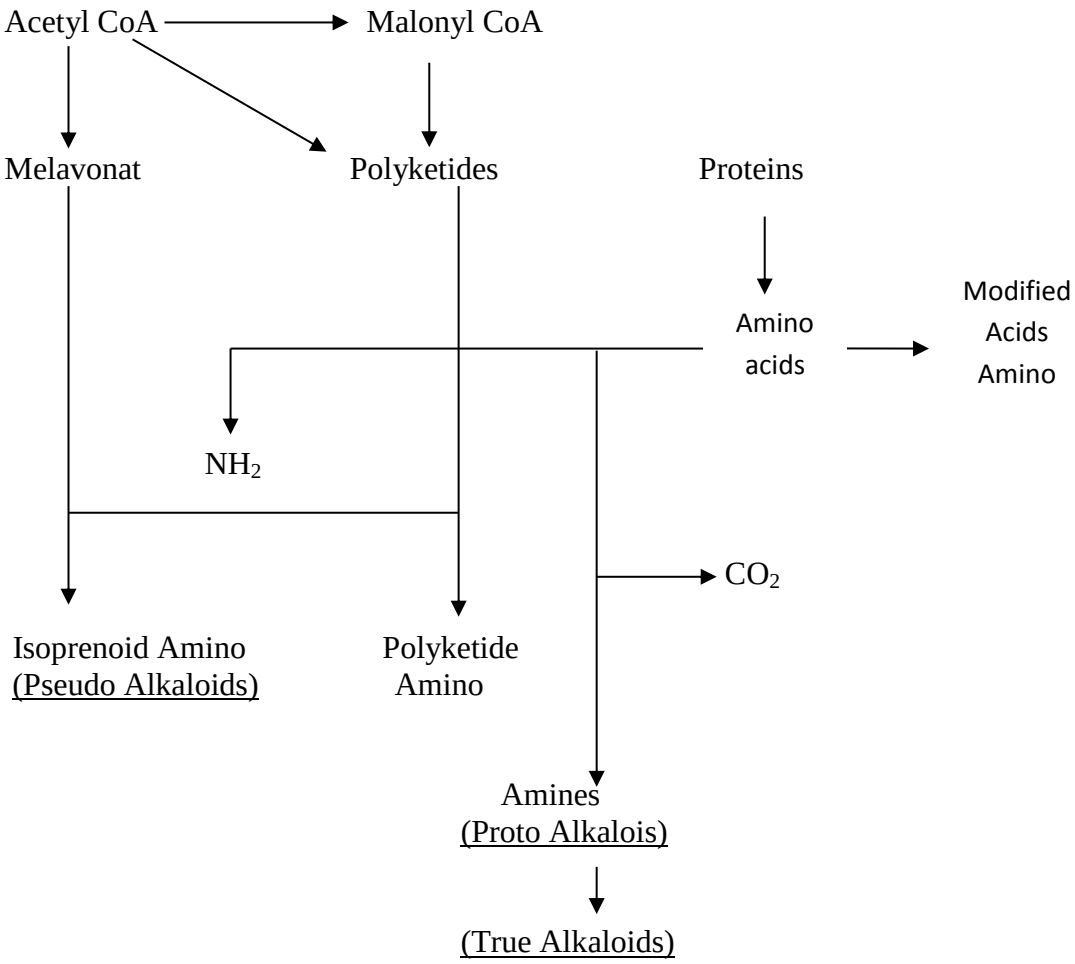
2.0 General

Family Rubiaceae is mainly distributed throughout the tropics. The family comprises of 10,700 species which are classified in about about 637 genera.[5] Many compounds have been isolated from Rubiaceae plants, as well as their biological activities which have been reported by numerous scientists, as many of the plants from this family are known for their medicinal values.

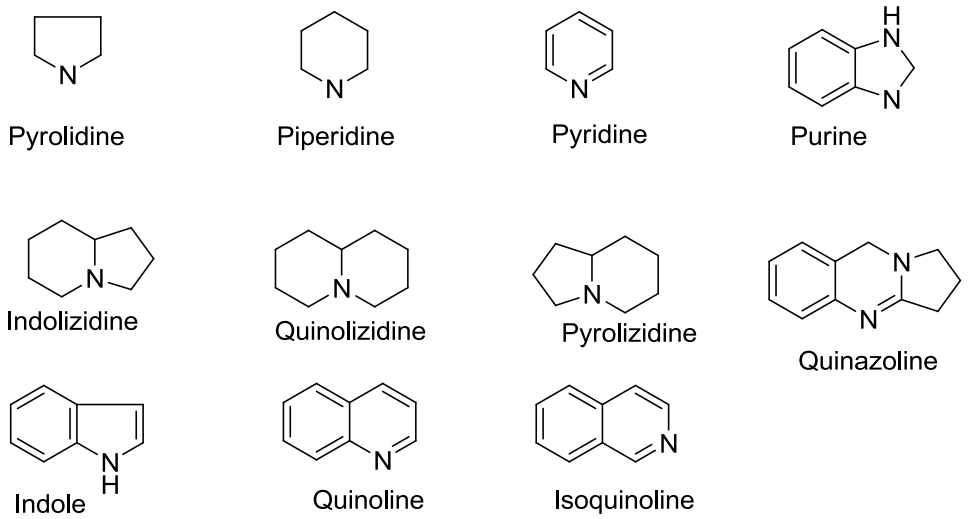
2.1 Alkaloids of Rubiaceae

Since the dawn of human civilization, alkaloids have been utilized as medicines, poisons, and magical potions. The term alkaloid was coined in 1819 by the pharmacist W. Meissner, which simply meant, "*alkali-like*".[17]

Classification of alkaloids are usually according to their common skeletal structure based on the metabolic pathway of the molecules (Scheme 2.1).[18] Scheme 2.2 shows the common alkaloid skeletons; piperidine, indolizidine, quinolizidine, pyridon, pyrrolidine, imidazole, quinazoline, quinoline, pyridine, sesquiterpene, indole, and purine.[19]



Scheme 2.1: Classification of Alkaloids with the Biogenesis Concept

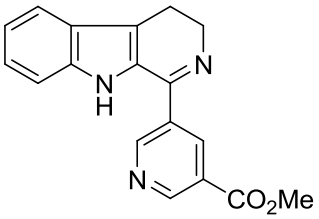


Scheme 2.2: Examples of Alkaloid Ring Skeletons

Alkaloids have been obtained from a number of genera in the Rubiaceae and the present knowledge has been reviewed.[20] Some alkaloid contents are used to prove a useful character for consideration by taxonomist attempting to resolve the classification of genera. They are including several alkaloid-yielding (produce alkaloid compound) such as *Adina, Anthecephalus, Nauclea, Sarcocephalus, Cephalantus, Mitragyna* and *Uncaria.*[21] The genus *Ochreinauclea* is closely linked botanically to the genus *Nauclea* and *Neonauclea*. In Malaysia they shared (Scheme 1.2) the same local name "bangkal". Throughout the world, there are only two *Ochreinauclea* species; *Ochreinauclea maingayii* and *Ochreinauclea missionis,* and this is the first phytochemical study on *Ochreinauclea maingayii.* *Naucle* and *Neonauclea* spesies usually produce indole alkaloids such as angustine **14**, parvine **7**, angustoline **24**, viscoside **28** and etc. Table 2.1 list the alkaloids isolated from previous study of *Naucle* and *Neonauclea* spesies.

Table 2.1: Alkaloids Isolated from Selected Species of Genus *Nauclea* and *Neonauclea*.

Genus	Species	Alkaloids	References
Nauclea	*Nauclea diderrichi*	Naucledine **5** Naucledерine **6**	[22,23]
	Nauclea parva	Parvine **7**	[24] [25]
	Nauclea orientalis	Nauclealines A **8** Nauclealines B **9** Naucleosides A **10** Naucleosides B **11** Strictosamide **12** Vincosamide **13** Angustine **14**	
	Nauclea latifolia	Nauclamides A **15** Nauclamides B **16** Nauclamides C **17** Nauclamides D **18** Nauclamides E **19** Nauclefine **20** Naucletine **21**	[26,27]
	Nauclea officinalis	Nauclefidine **22** Naucledinal **23** Angustoline **24**	[28,29]
Neonauclea	*Neonaulea sessilifolia*	Neonaucleoside A **25** Neonaucleoside B **26** Neonaucleoside C **27** Viscoside **28** Strictosidine **29**	[30]
	Neonauclea schlechteri	Gambirine **30**	[31]



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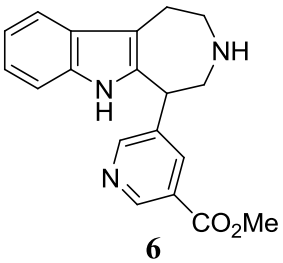

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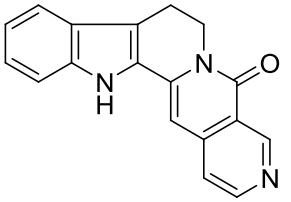

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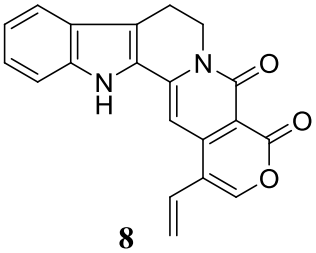

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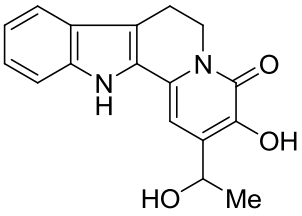

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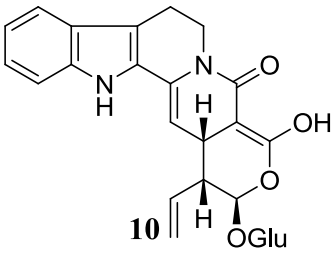

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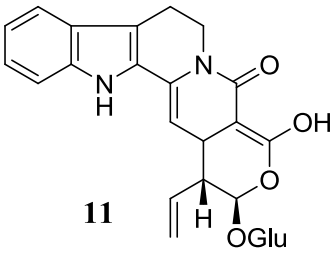

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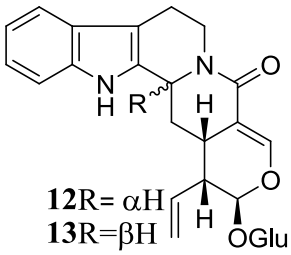
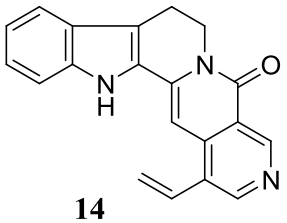


12R= αH
13R=βH



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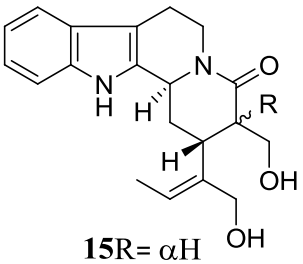
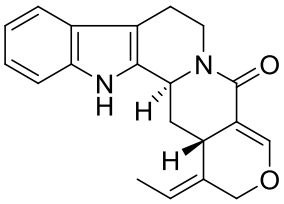


15R= αH
16R=βH



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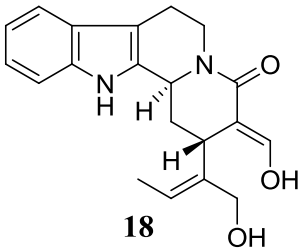

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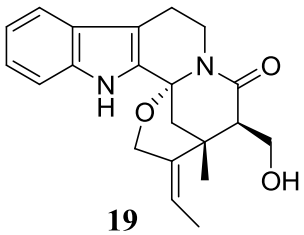

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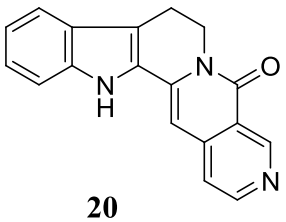

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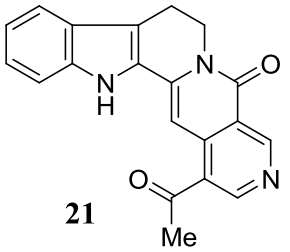

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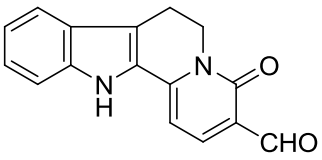

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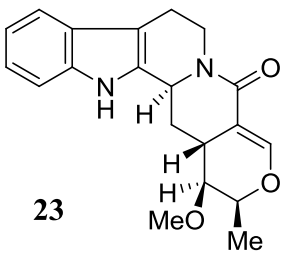

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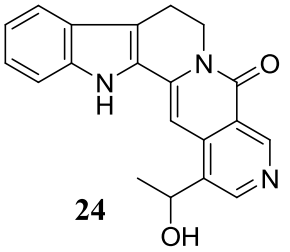

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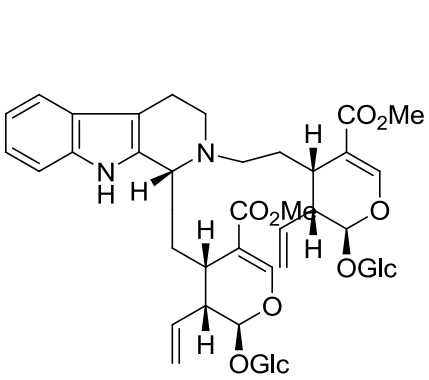

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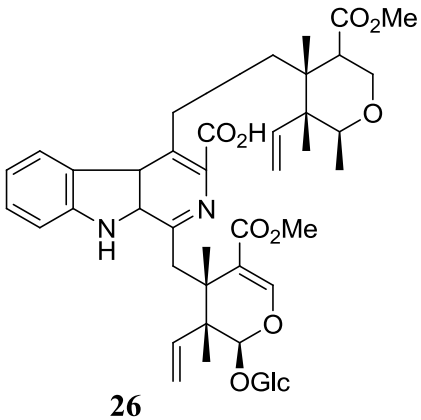

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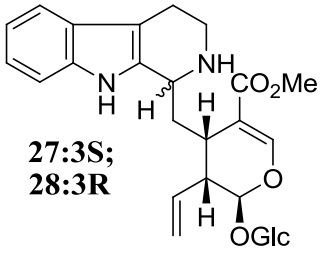
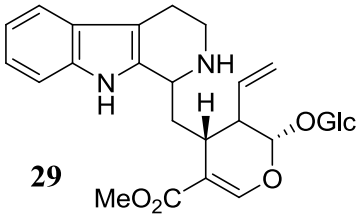


27:3S;
28:3R



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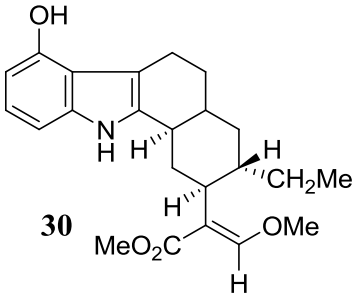
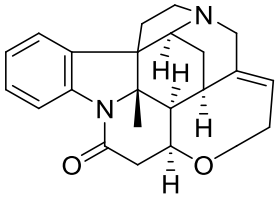


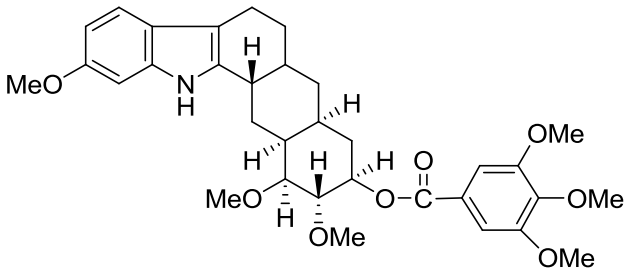
30

2.2 Indole Alkaloids in Rubiaceae

Alkaloids with an indole nucleus make up an extensive and complex group.[32] They include physiologically active compounds such as strychnine **31,** a convulsant poison, reserpine **32,** a hypotensive sedative agent, and lysergic acid **33**, the dimethylamide derivative of which is the powerful hallucinogen LSD capable of inducing symptoms similar to schizophrenia. An illustration of the complexity of the structures encountered in this field is the antileukaemia alkaloids vincristine **34**, vinblastine **35**, vinrosidine **36**, and vinleurosine **37**. These alkaloids form some of the most potent drugs available to man for the treatment of a variety of cancers.[33]



31



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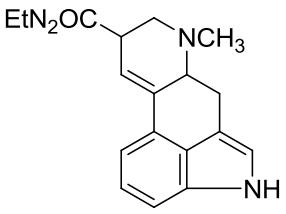

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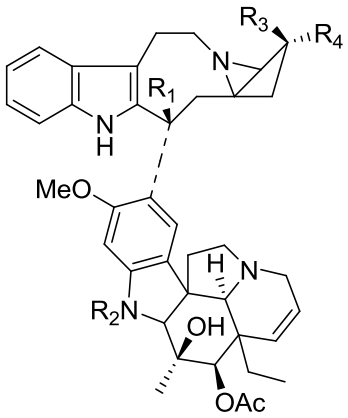
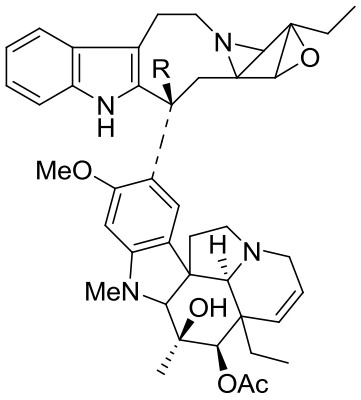
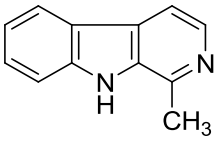


34: R_1=COOMe, R_2=CHO, R_3= OH, R_4=Et
35: R_1=COOMe, R_2=Me, R_3= OH, R_4=Et
36: R_1=COOMe, R_2=CHO, R_3= Et, R_4=OH

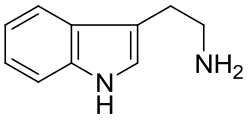


37

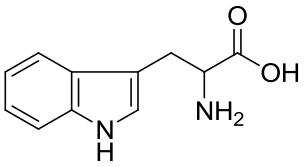
Most of indole alkaloids with obviously related structures are found in many plants especially from the family Apocynaceae, Lagoniaceae, Euphorbiaceae and also Rubiaceae.[20] Indole alkaloids can be divided into two main classes. First are the simple indole alkaloids that possess only indole nucleus, for example harmane **38**. The second class includes two structural units: tryptamine **39** or tryptophan **40** with indole nucleus and a C9 or C10 monoterpene moiety derived from secologanin **41**.



38



39



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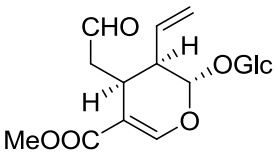
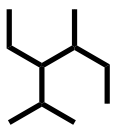
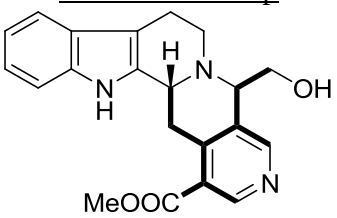
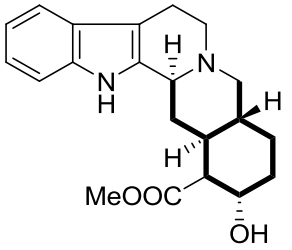


41

Indole alkaloids in Rubiaceae plants can be further classified according to their structural characteristic of their skeletons. There are Class I, II, III, IV (divided to non-trytophan indole alkaloids, non-isoprenoid tryptophan alkaloids and isoprenoid tryptophan alkaloids) and Class V (binary indole alkaloids).[34] But majority of the indole alkaloids of Rubiaceae are from classes I, II, and IV.

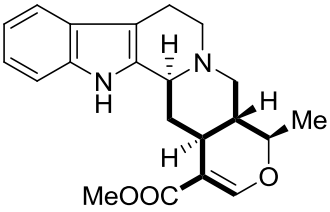
Table 2.2: Classification of Indole Alkaloids

Class of Indole Alkaloids	**Example**
Class I 	Cadamine Group  Cadamine (**42**) Yohimbine Group



Yohimbine (**43**)

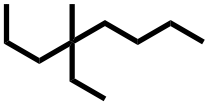
Ajmalicine Group

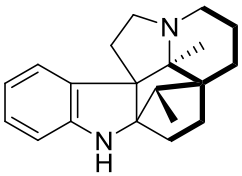


Ajmalicine **44**

Class II

Vindoline Group

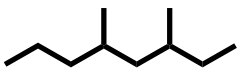


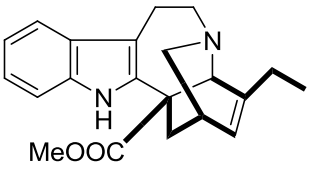


Tuboxinine **45**

Class III

Catharanthine Group



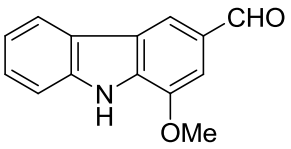


Catharanthine **46**

Class IV

Non-trytophan

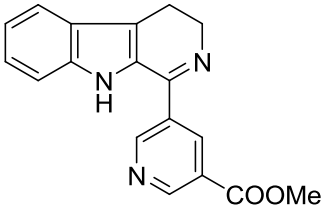
Murrayanine Group



Murrayanine **47**

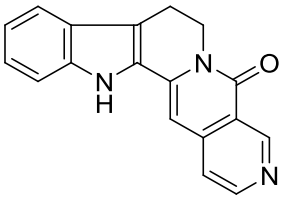
Non-isoprenoid tryptophan

Indolopyridine Group



Indolopyridine A **48**

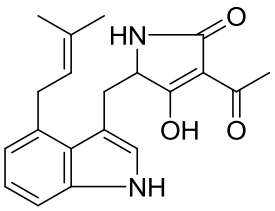
Nauclefine Group



Nauclefine **20**

Isoprenoid tryptophan

B-Cyclopiazonic acid group

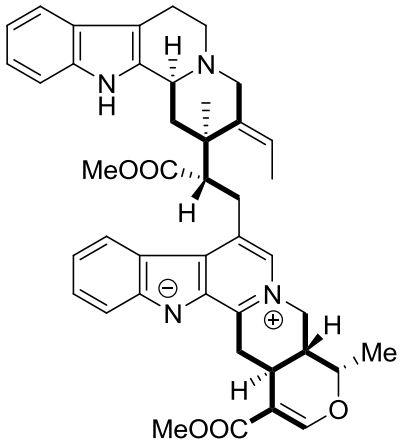


β-Cylopiazonic acid **49**

Class V

(binary indole alkaloids)

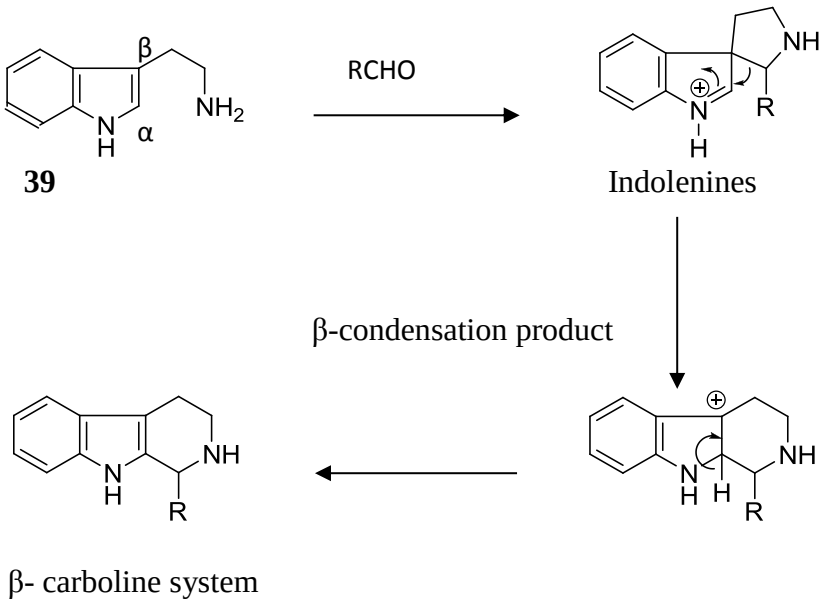
I-I Group



Serpentinine **50**

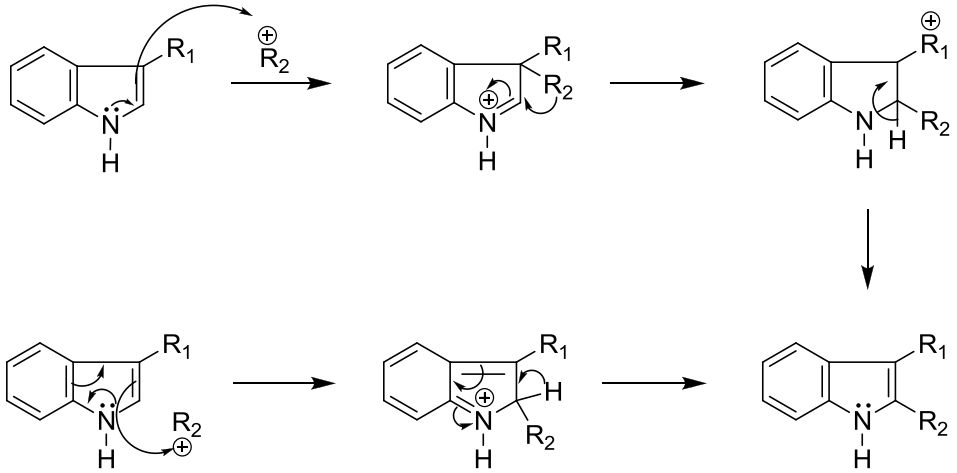
2.3 Biosythesis of Indole Alkaloids

The basic structure of indole alkaloids can be seen to be derived from the Mannich condensation of tryptamine **39** with aliphatic aldehyde having nine or ten carbon at the α- or β- positions of indole nucleus. Perkin and Robinson was the first to suggest that aromatic portion present in the indole alkaloid is derived from tryptophan which has undergone decarboxylation to tryptamine **39**.[35] Experimental evidence for such decarboxylation has been obtained by Battersby and co-workers.[36] Tryptophan **40** or tryptamine **39** could condense with an appropriate aldehyde to form a Schifff base which could be attacked intramolecularlly by the β- position of the indole nucleus to afford the corresponding indolenines (Scheme 2.3).

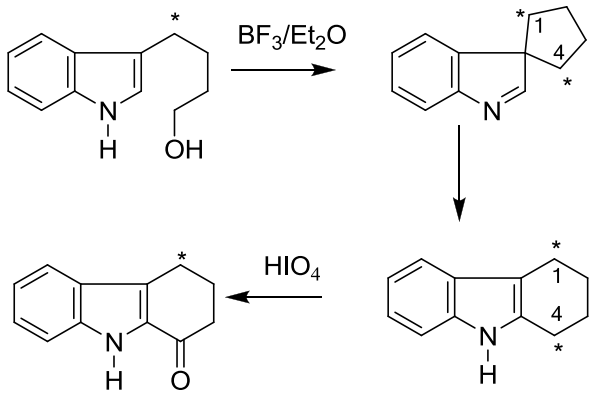


Scheme 2.3: Biosynthetic Hypothesis for the Construction of the β-carboline System from Tryptamine **39**

An examination of the reaction mechanism shows that such a direct attack would involve the generation of an intermediate with a disturbed π-electron cloud on the benzene ring which would be energetically unfavourable. An alternative pathway avoiding the generation of such a destabilized structure would be first the formation of the indolenine from β-condensation, which could then rearrange by migration of the R_2 group to the α-position (Scheme 2.4). Experimental evidence that support this alternative pathway has been provide by Jackso and co-workers in Scheme 2.5.[37] The position of tritium exhibited at asterisked carbon, where the cyclic alcohol oxidized with periodic acid that gave the ketones with half radioactivity to precede the formation of symmetrical spirocyclic indolenine which make position 1 and 4 equivalent and distributed the radioactivity equally in the tetrahydrocarbazole.

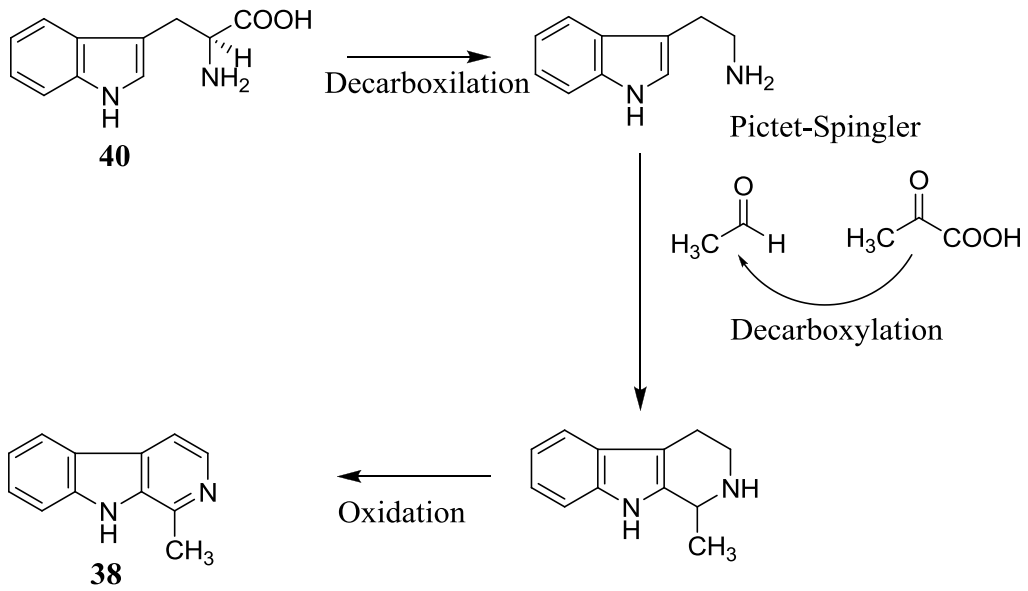


Scheme 2.4:The Indolenine Pathway leading to the α–carboline System



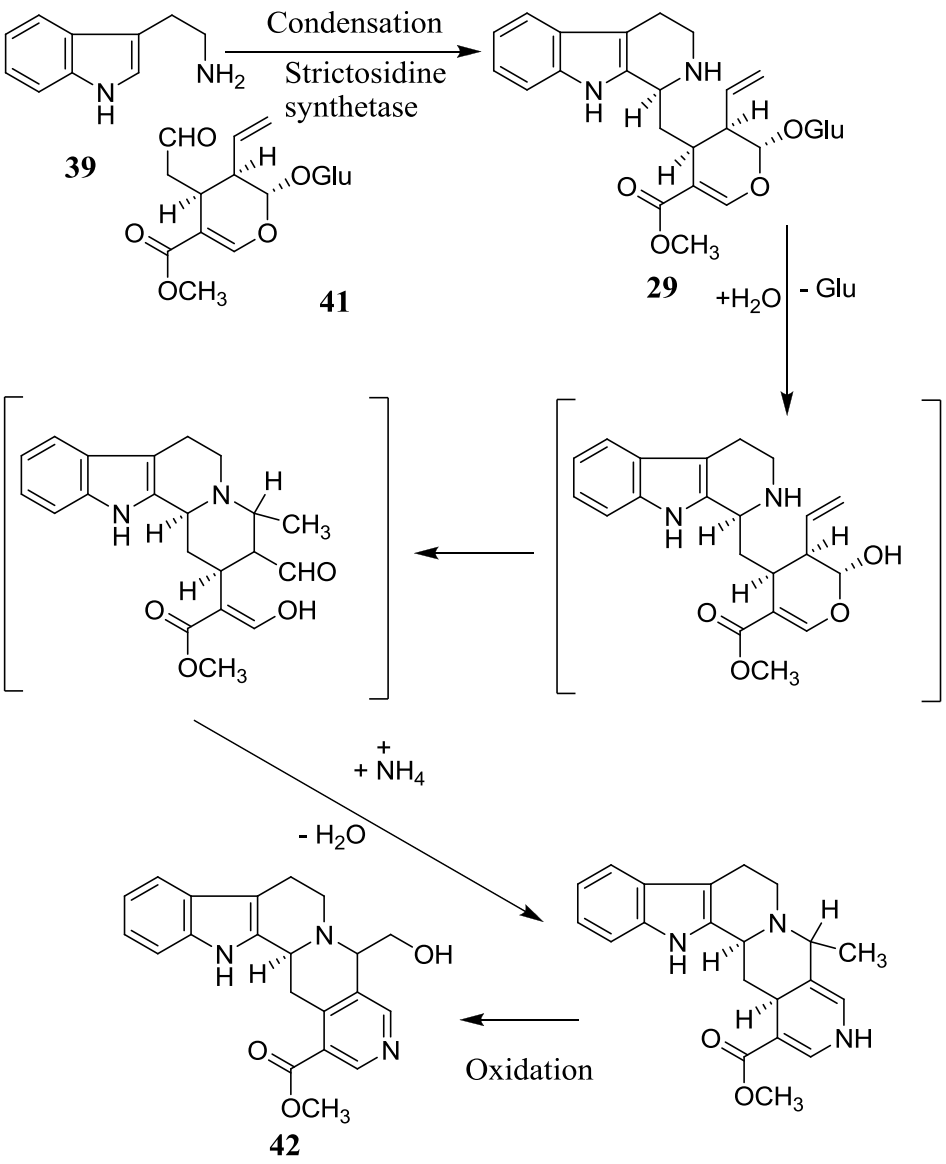
Scheme 2.5: Isotopic Labeling Experiments Consistent with the Indolenine Mechanism

Harmane **38** is example that representing of an indole-ring containing alkaloid that is derived from tryptophan **40** via decarboxylation and Pictet-Spingler reaction (Scheme 2.6). The biosynthetic route to harmane **38** is relatively straight forward: decarboxylation of tryptophan **40** and a subsequent Pictet-Spingler reaction with acetaldehyde (CH_3CHO) give tetrahedron derivatives, from which harmane **38** is formed by oxidation.[38]



Scheme 2.6 : Biosythesis of Harmane **38**

Cadamine **42** is an example of a more structurally complex indole alkaloid which is also formed from tryptamine **39** and secologanin **41** via condensation process in the presences of enzyme strictosidine synthetase to form strictosidine **29**. Glycosidase enzyme converts strictosidine **29** to a series of reactive intermediates via hydrolysis and to formed cadamine **42** by oxidation (Scheme 2.7).[39]



Scheme 2.7: Biogenetic pathway for Cadamine **42**

2.4 Spectroscopic Features of Indole Alkaloids

Spectroscopic methods are now employed in the solution of almost all structural problems in organic chemistry. Basically there are five invaluable methods used for structure elucidation, which are:

i) Ultraviolet spectroscopy to detect the conjugated systems

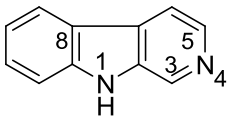
ii) Infrared spectroscopy to find out the functional groups

iii) NMR spectroscopy to detect changes in the alignment of nuclear magnets in strong magnetic field

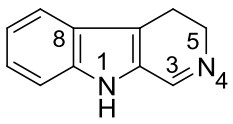
iv) Mass spectroscopy for measures the mass-to-charge ratio of organic ions

v) Optical rotary dispersion to measure the change in rotary power of molecules

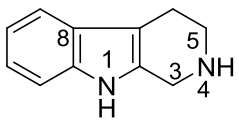
2.4.1 β-Carboline Type



51



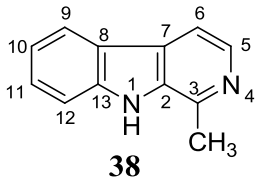
52



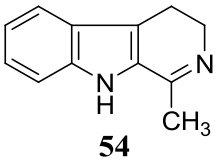
53

The aromatic proton of an unsubstitued indole β-carboline **51** skeletons at position C-5, C-6, C-9, C-10, C-11 and C-12 are located at upfield region between δ_H 7.00 – 8.90 and cannot be easily differentiated from one another. Usually hydrogen at position C5 is found relatively downfield with respect to other due to the neighboring N4-atom. Proton H-5 and H-6 normally give a characteristic AB system signal at about δ_H 7.90 – 8.90 with coupling constant about 5.0-6.0 Hz. The small coupling constant *J* value is due to the adjacent N-4 atom. The dihydro beta carboline **52** is slightly different with beta carboline. Beta carboline is fully conjugated while dihydro β carboline **52** is

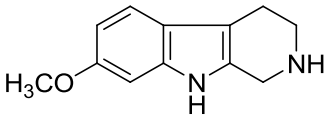
absent of double bond at position C-5 and C-6 in ring C, thus it has aliphatic proton and resonates at higher field between δ_H 2.50-4.20. Tetrahydro β-carboline **53** is absence all double bond at ring C. It has aliphatic protons at position C-5, C-6 and C-3. The signal appears at higher field at region δ_H 2.50-4.50, usually as multiplet peak. Therefore, proton H-3 will appear as methines if they have another ring or subtituted attach at the skeleton. The Table 2.3 and 2.4 below show the example of chemical shifts for some beta carboline of indole alkaloids, such as harmane **38**[40,41], harmalan **54** and tetrahydronorharmine **55**.



38



54



55

Table 2.3: ^{1}H-NMR data (δ/ppm)Hz of β-carboline ;harmane **38**, harmalan **54** and tetrahydronorharmine **55**.

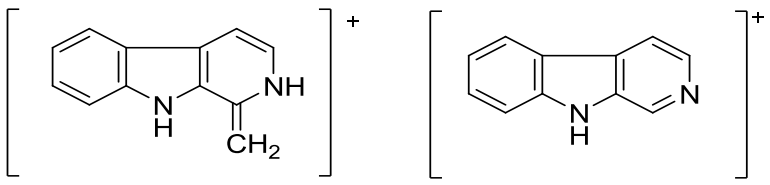
Position H	Harmane **38** [Seki, 2000][41]	Harmalan **54** [Parker, 2004][42]	Tetrahydronorharmine **55** [Samoylenko, 2010][43]
2	-	-	-
3	-	-	-
5	8.26 *d (J=5.5)*	3.97 *t* (*J*=7.2)	3.30 *m*
6	7.94 *d (J=5.5)*	3.32 *m*	2.81 *m*
7	-	-	
8	-	-	
9	8.21 *d* (*J*=7.7)	7.74 *d* (*J*=7.8)	7.10 *d* (*J*=7.5)
10	7.25 *t* (*J*=7.7)	7.21 t (*J*=7.2)	6.49 *d* (*J*=7.5)
11	7.56 *t* (*J*=7.7)	7.47 *m*	
12	7.65 *d* (*J*=7.7)	7.51 *d* (*J*=8.4)	6.67 *s*
13	-	-	-

Table 2.4: ^{13}C-NMR data (δ/ppm) of β-carboline; harmane **38**, harmalan **54** and tetrahydronorharmine **55**.

Position C	Harmane **38** [Seki, 2000][41]	Harmalan **54** [Parker, 2004][42]	Tetrahydronorharmine **55** [Samoylenko, 2010][43]
2	141.7	155.0	124.0
3	134.6	129.6	41.0
5	138.6	43.6	42.6
6	113.0	18.8	18.5
7	128.5	117.8	105.8
8	122.1	120.0	120.8
9	122.0	124.9	118.2
10	120.3	120.2	109.2
11	117.7	124.8	120.9
12	128.4	112.3	94.5
13	140.2	136.5	137.8

Mass spectroscopy

The mass spectrum of indole β-carboline alkaloids usually shows a peak at *m/z* 182. This base peak is typical, and in addition a peak at *m/z* 168 is characteristic of β-carboline structures.



m/z 182 *m/z* 168

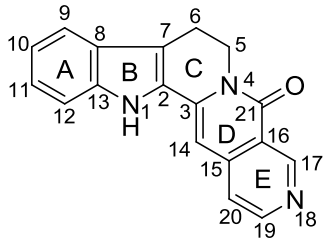
UV spectroscopy

Indole has a special characteristic ultraviolet maxima absorptions at 225 and 270 nm.[44] The maximun absorption is increased due to the existence of indoline chromophore or a highly conjugated system of indole.

IR spectroscopy

The IR spectrum of indole β-carboline alkaloids usually shows broad absorption bands due to OH/NH groups (3420 and 3172 cm^{-1}) and typical absorption for aromatic rings (1600-1450 cm^{-1}).

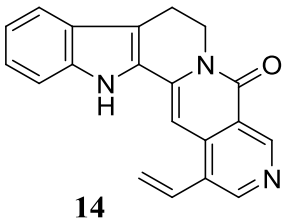
2.4.2 Nauclefine Type



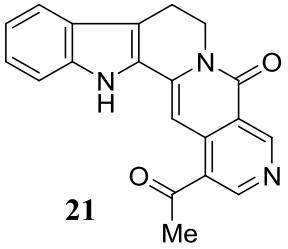
20

Nauclefine **20** represents the basic indolopyridinoquinolizidinone skeleton.[24,45] In ^{1}HNMR spectrum, four aromatic protons in ring A appeared at region δ_H 7.00-8.00 ppm attributable to H-9 (*d, J*=8.0Hz), H-10 (*t, J*=8.0), H-11 (*t, J*=8.0) and H-12 (*d, J*=8.0). H-14 of ring D was revealed as a singlet at region δ_H 6.00-8.00 indicating that a double bond formed between C-15 and C-16. In addition, the C-6 and C-5 methylene proton signals normally appeared at δ_H 3.00-4.00 and 4.00-6.00 respectively. In ring D,

the carbonyl carbon C-21 resonated at δ_c 161.0. Therefore, the signal of C-17 and C-19 protons usually appeared as singlets at δ_H 8.00-10.00. The Tables 2.5 and 2.6 show the comparison of ^{1}HNMR and ^{13}CNMR spectroscopic data between nauclefine **20**, angustine **14** and naucletine **21**.



14



21

Table 2.5: ^{1}H-NMR data (δ/ppm)Hz of Nauclefine type ;nauclefine **20**, angustine **14** and naucletine **21**.

Position H	Nauclefine **20** [Erdelmier, 1992][46]	Angustine **14** [Abreu, 1998][47]	Naucletine **21** [Liew, 2012][40]
5	4.52 *t (J=6.6)*	4.40 *t*(*J*=6.0)	4.39 *t* (*J*=6.9)
6	3.18 *t (J=6.6)*	3.15 *t* (*J*=6.0)	3.12 *t* (*J*=6.9)
9	7.62 *d* (*J*=7.9)	7.63 *d* (*J*=8.0)	7.65 *d* (*J*=8.0)
10	7.17 *t* (*J*=7.5)	7.12 *t* (*J*=7.2)	7.07 *m*
11	7.31 *t* (*J*=7.5)	7.29 *m*	7.23 *m*
12	7.44 *d* (*J*=7.9)	7.49 *d* (*J*=8.0)	7.45 *d* (*J*=8.1)
14	6.76 *s*	7.29 *m*	7.73 *s*
17	9.49 *s*	9.23 *s*	9.21 *s*
19	8.57 *d* (*J*=5.5)	8.87 *s*	9.41 *s*
20	7.36 *d* (*J*=5.5)		
22		6.06 *d* (*J*=17.6)	
		5.63 *d* (*J*=10.8)	
23		7.29 *m*	2.71 *s*

Table 2.6: ^{13}C-NMR data (δ/ppm) of Nauclefine type; nauclefine **20**, angustine **14** and naucletine **21**.

Position C	Nauclefine **20** [Abreu, 1998][46]	Angustine **14** [Abreu, 1998][47]	Naucletine **21** [Liew, 2012][40]
2	127.6	126.8	127.4
3	137.2	136.9	140.8
5	40.3	40.4	40.7
6	19.3	19.2	19.8
7	114.8	114.8	116.9
8	125.4	125.5	125.7
9	119.7	119.9	119.3
10	119.9	119.9	119.9
11	124.5	124.6	120.9
12	112.0	112.0	112.0
13	138.6	138.5	139.0
14	97.0	93.8	95.6
15	141.6	139.0	141.1
16	119.0	119.8	117.1
17	150.5	149.7	154.0
19	151.0	147.7	155.4
20	119.0	127.8	138.8
21	161.1	161.1	161.6
22		119.8	199.6
23		130.2	29.3

UV spectroscopy

The UV usually revealed the characteristic of maxima absorption of indole chromophore. Table 2.7 showed the maxima absorbance of nauclefine **20**, aungustine **14** and naucletine **21**.

Table 2.7: UV maxima Absorption of nauclefine **20,** angustine **14** and naucletine **21**

Compounds	UV (nm)	Chromophore	Reference
Nauclefine **20**	389, 371, 287, 259	Indole	[47]
Angustine **14**	400, 380, 305, 292, 255, 222	Indole	[45]
Naucletine **21**	404, 312, 260, 216	Indole	[27]

IR spectroscopy

The IR spectrum give broad absorption bands due to OH/NH groups at around 3300 cm^{-1} and typical absorption for aromatic ring and C=O group at around 1600 cm^{-1}.

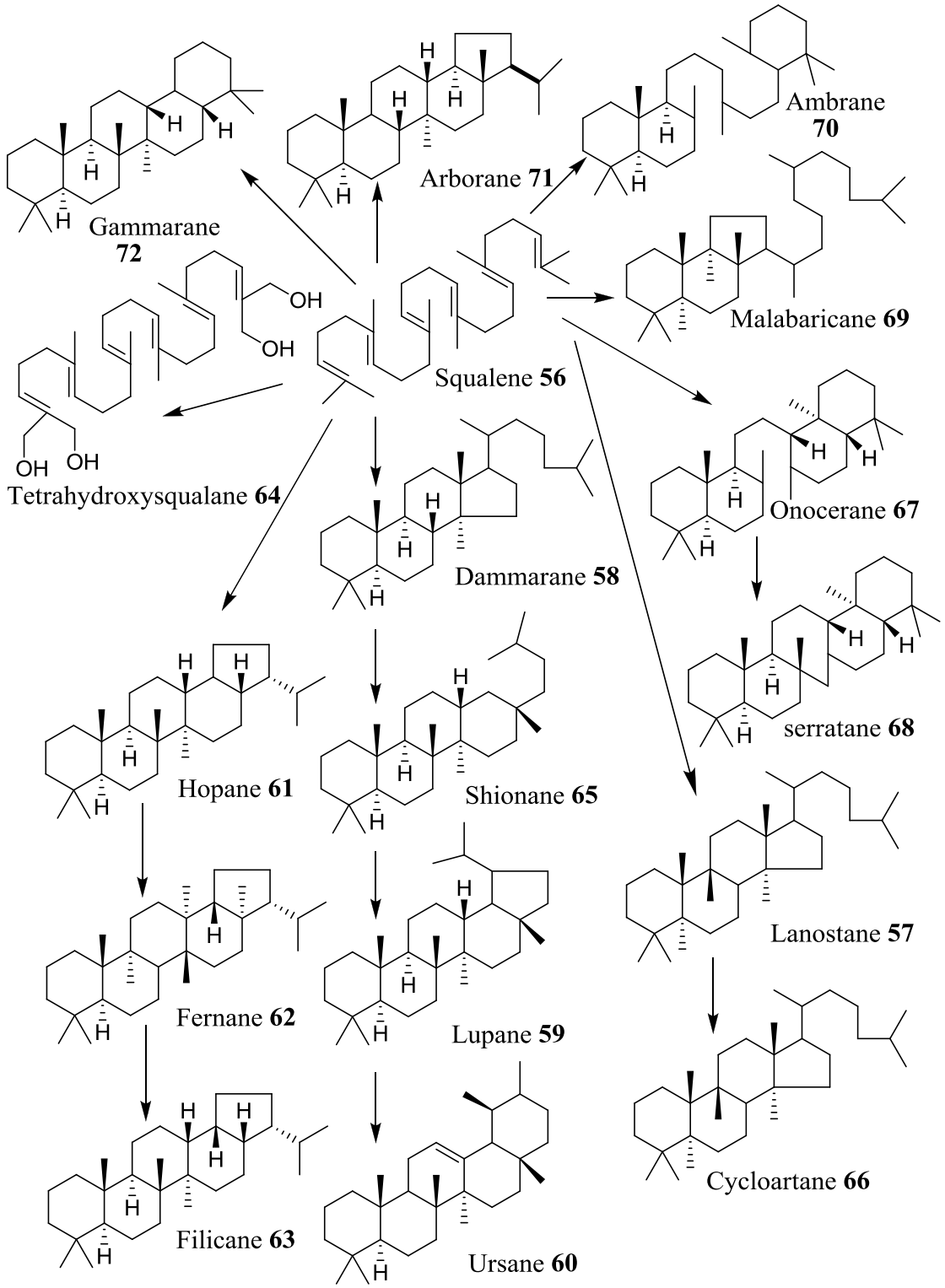
2.5 Triterpenoid Groups

Triterpenoid is a group of terpene that comprises of thirty carbon atoms. The triterpenoids; over 4000 compounds built upon over 40 different skeletons, form the largest group among the terpene classes, and are widely distributed in the plant kingdom, either in the free state or as esters or glycosides.

2.5.1 Biosynthesis of Triterpenoids

Triterpenoids consist of various groups, such as squalene **56**, lanostane **57**, dammarane **58**, lupane **59**, ursane **60**, and hopane **61**, which are the most well-known triterpenoid skeletons (Scheme 2.8). These skeletons exist as a result of cyclization, rearrangement and degradation. There are however several pathways known for the

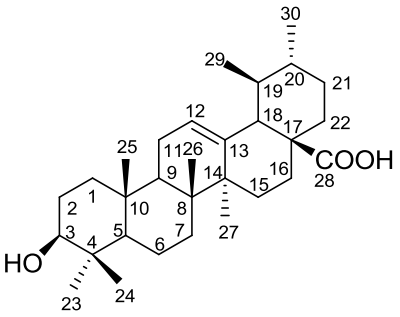
biosynthesis of triterpenoids, leading to different types of triterpenoid skeletons. Several skeletons have been found to undergo ring cleavage leading to seco skeletons, hemologation (leading to homo and bis homo skeleton), degradation and minor rearrangement to related skeleton. [48,49]



Scheme 2.8: Cyclization of Squalane to Various Triterpene Skeleton.

2.5.2 Ursane Type

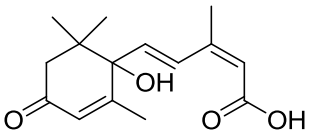
Ursane type is also called α-amyrin.[50] This skeleton type has 30 carbons with pentacyclic rings. Ursolic acid **73** (3β-hydroxy-urs-12-en-28-oic acid) was the basic triterpenoid compound which exist widely in nature in free acid form as aglycones those representing ursane type triterpenoid saponins.[51] It has one olefinic carbon at C12 and C13 usually observed at δ 138 and 128 in ^{13}CNMR spectra.[52]



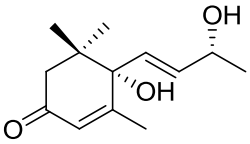
73

2.6 *Nor*-isoprenoid

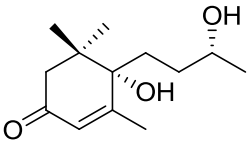
Nor-isoprenoid or also called "carotenoid-like" is compound with thirteens carbon atoms, are neither abundant nor usual as natural products.[53] Their biogenetic origin is yet uncertain. Some authors have suggested that they may be biosynthesized from (+)-abcisic acid by oxidative removal of two terminal carbon atoms. Blumenol A, B and C were the first *nor*-isoprenoid that extracted from the leaves *Podocarpus blumei*.[54, 55]



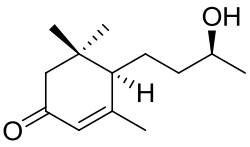
(±) Abcisic acid **74**



Blumenol A **75**



Blumenol B **76**



Blumenol C **77**

CHAPTER 3

RESULTS

AND

DISCUSSION

RESULTS AND DISCUSSION

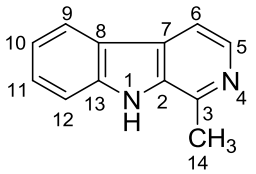
3.0 General

The bark and leaves from *Ochreinauclea maigayii* has been studied. The extraction was carried out using conventional method with various solvent systems such as hexane, dichloromethane and methanol and the isolation processes were carried out using the conventional methods such as extensive column chromatography on silica gel followed by preparative thin layer chromatography. The structural elucidation as established through several spectroscopic methods; 1D and 2D-NMR (^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMQC, HMBC), UV, IR, MS and also by comparison with the literature data.

3.1 Isolation and Structural Elucidation of Compounds from *Ochreinauclea maingayii* (Hook.F.) Ridsd.

The extraction of the bark and leaves from dichloromethane yielded compound those are harmane **38,** naucledine **5,** cadamine **42**, neonaucline **79**, isodihydrocadambine **78**, ursolic acid **73**, blumenol A **75,** benzamide **80** and cinnamide **81.**

3.1.1 Compound A: Harmane 38



38

Compound **A** was isolated as a brownish amorphous solid. In the UV spectrum, absorption maxima were observed at 347, 334, 287, 239, 234, and 212 nm.[41] The IR spectrum of this alkaloid showed absorption peak at 3125 cm^{-1} indicating the presence of NH group of the indole alkaloid. The LCMS-IT-TOFF mass spectrum (Figure 3.1) revealed a $[M+H]^+$ peak at *m/z* 183.0921, thus suggesting a molecular formula of $C_{12}H_{10}N_2$ with nine degrees of unsaturation which the presence of three rings and suggested six double bonds.

The ^1H NMR (Figure 3.2) spectrum showed four vicinal aromatic hydrogen signals δ 8.09 (*d*, *J*= 7.8 Hz, H-9), 7.52-7.50 (*m*, H-12), 7.26-7.23 (*m*, H-10), 7.52-7.50 (*m*, H-11), characterizing a *β*-carboline skeleton moiety. A pair of doublet peaks at δ 8.33 and 7.80, integrating for two protons could be assigned to H-5 and H-6 respectively. A broad singlet signal of N-H appeared at δ 8.70 (N-1) and a methyl singlet signal appeared at δ 2.81 which refer to the methyl attached to C-3.

The COSY spectrum (Figure3.4) showed cross peaks between; H-5 (δ 8.33) and H-6 (δ 7.80); H-9 (δ 8.09) and H-10 (δ 7.26-7.23); H-10 (δ 7.26-7.23) and H-11 (δ 7.52-7.50). The ^{13}C NMR spectrum (Figure 3.3) is in agreement with the molecular formula suggested from the mass spectrum, indicating for all 12 carbons; five quaternary carbons, six methine carbons, and one methyl carbon. The most upfield signal at δ 21.0 belongs to the C-14 methyl carbon. The HMQC and HMBC (Figure 3.5 and 3.6)

experiments established the complete assignments of all carbons and protons that summarized in Table 3.1.

Finally, comparison of all obtained data with the literature values confirmed[41, 42, 56] that compound **A** is the known alkaloid harmane **38**.

Table 3.1: ^{1}HNMR, ^{13}CNMR (δppm) spectral data of Compound **A** in $CDCl_3$

Position	^{1}H (δ_H, $CDCl_3$,Hz)	^{13}C (δ_C, $CDCl_3$)
N-H	8.70 *s*	-
2		142.0
3		134.5
5	8.33 *d* *J*=5.4	138.0
6	7.80 *d* *J*=5.4	112.6
7		128.2
8		122.2
9	8.09 *d* *J*=7.8	122.0
10	7.26-7.23 *m*	120.0
11	7.52-7.50 *m*	111.4
12	7.52-7.50 *m*	128.0
13		140.5
14	2.81 *s*	21.0

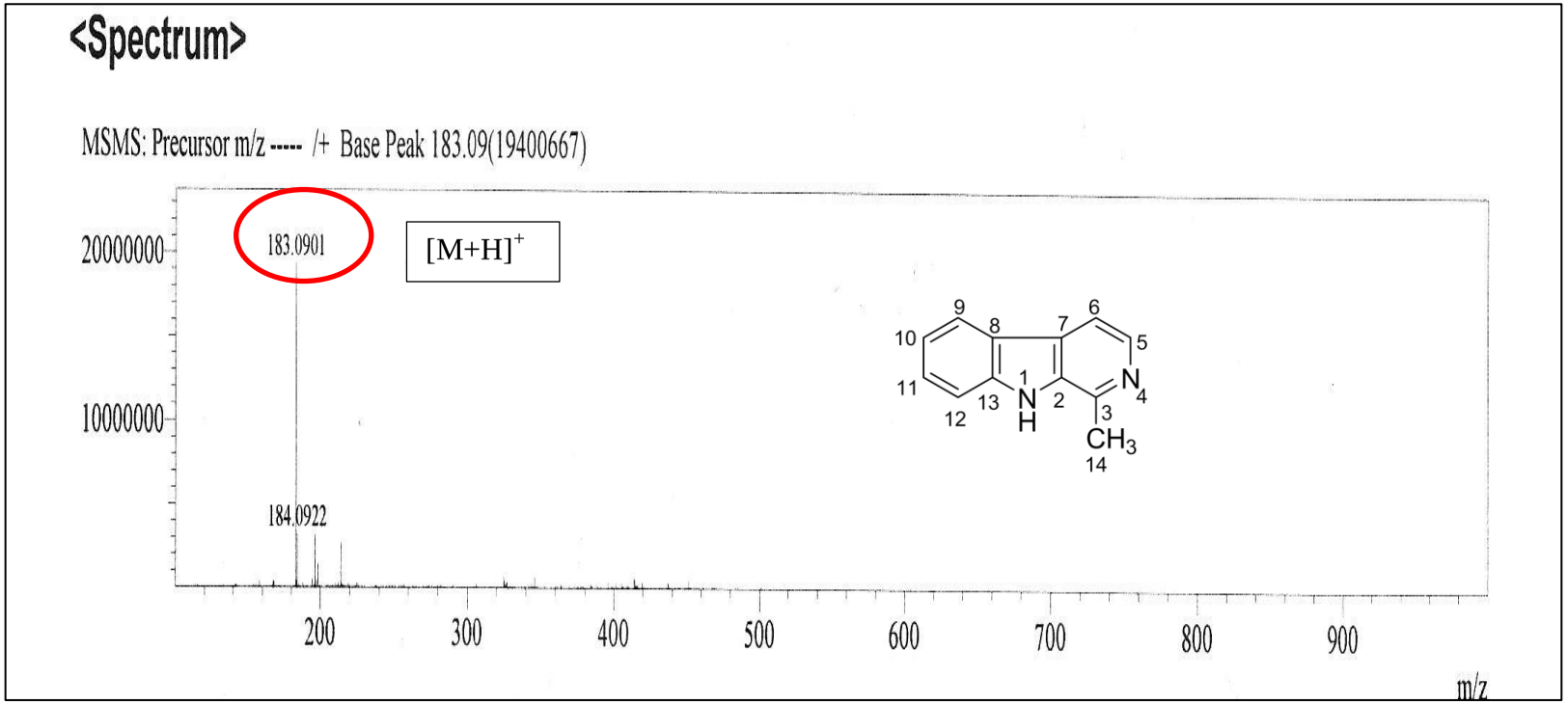


Figure 3.1: LCMS-IT-TOFF Spectrum of Compound **A**

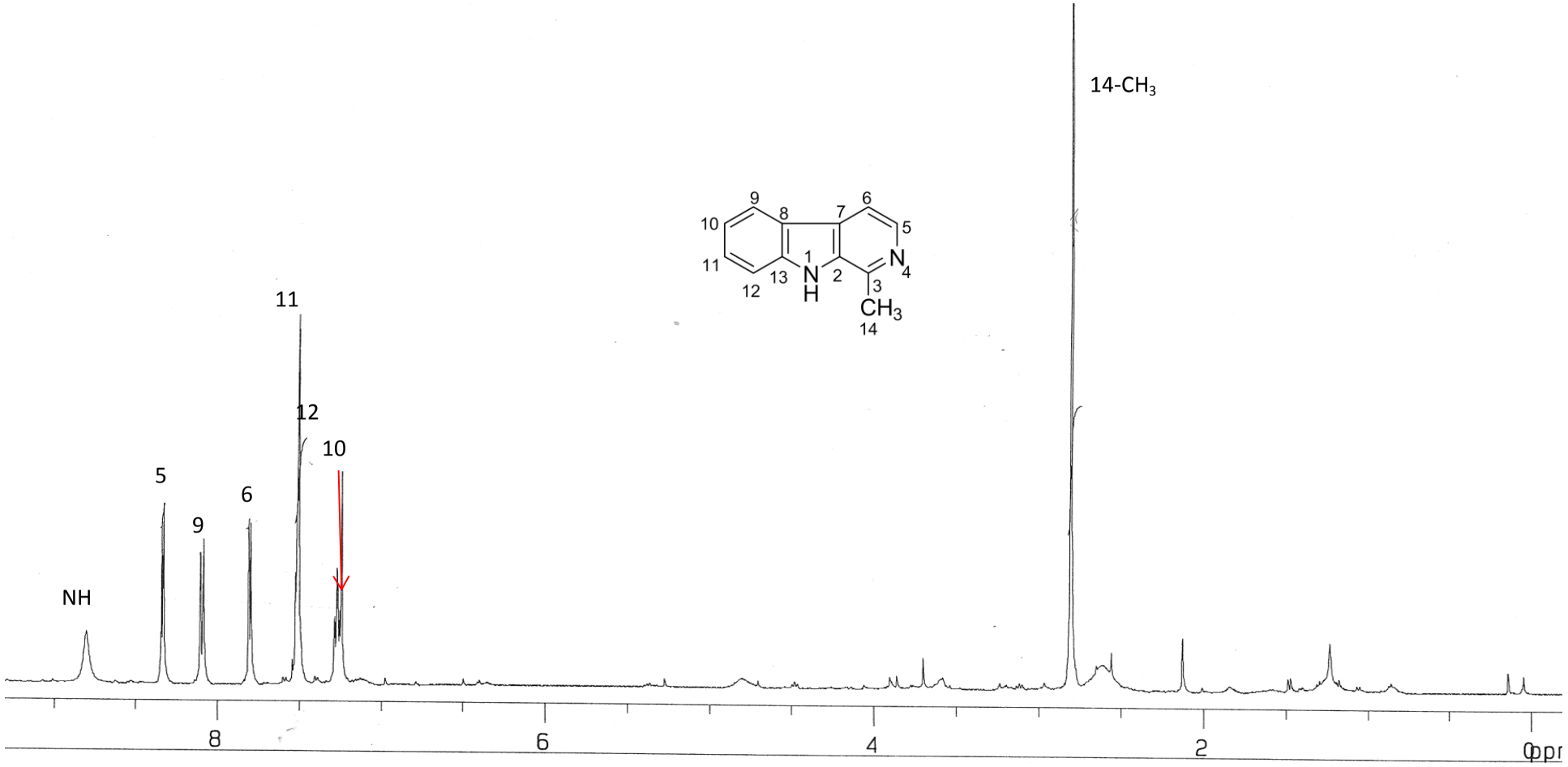


Figure 3.2: ^{1}HNMR Spectrum of Compound **A**

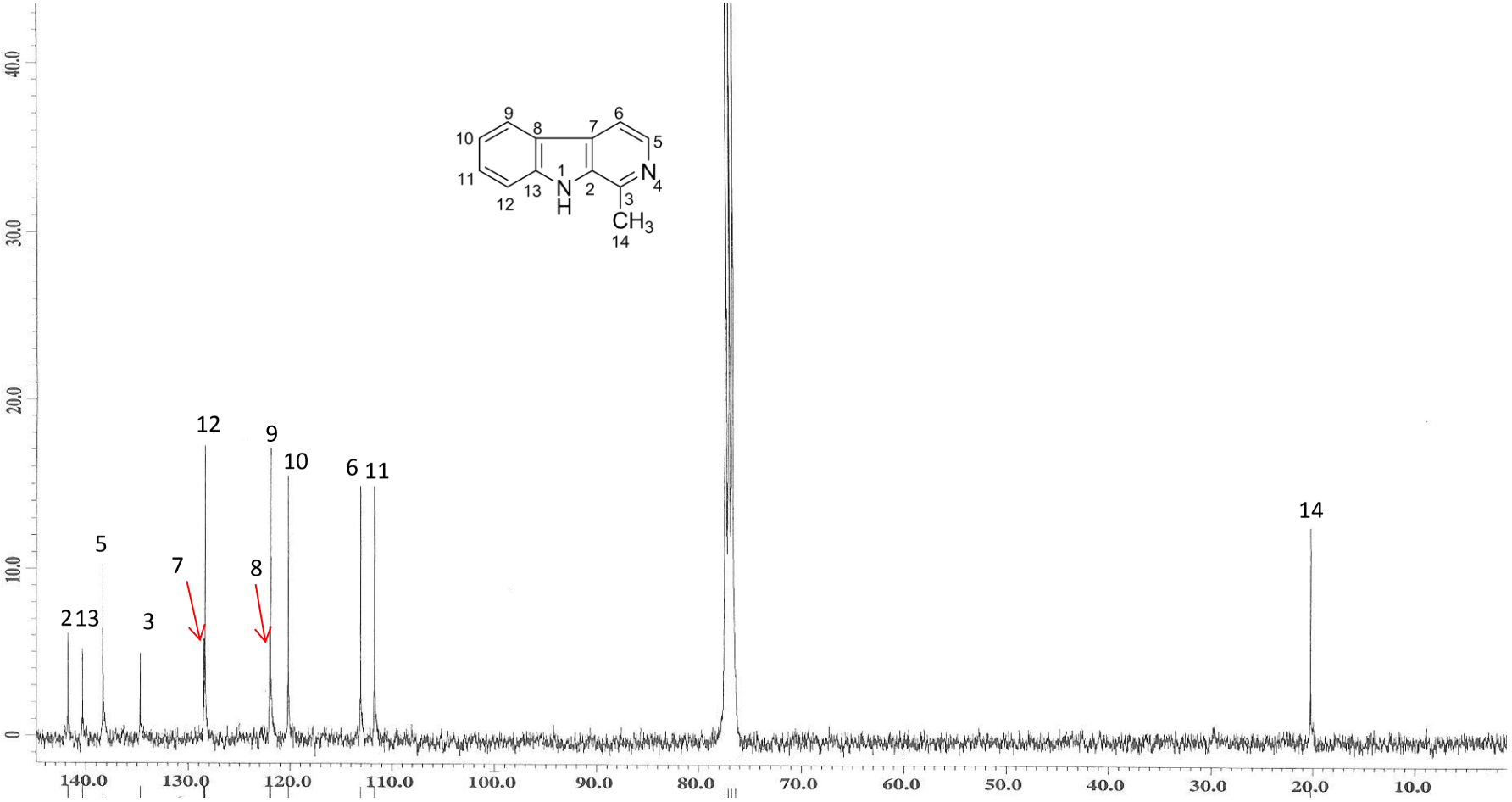


Figure 3.3: ^{13}CNMR Spectrum of Compound **A**

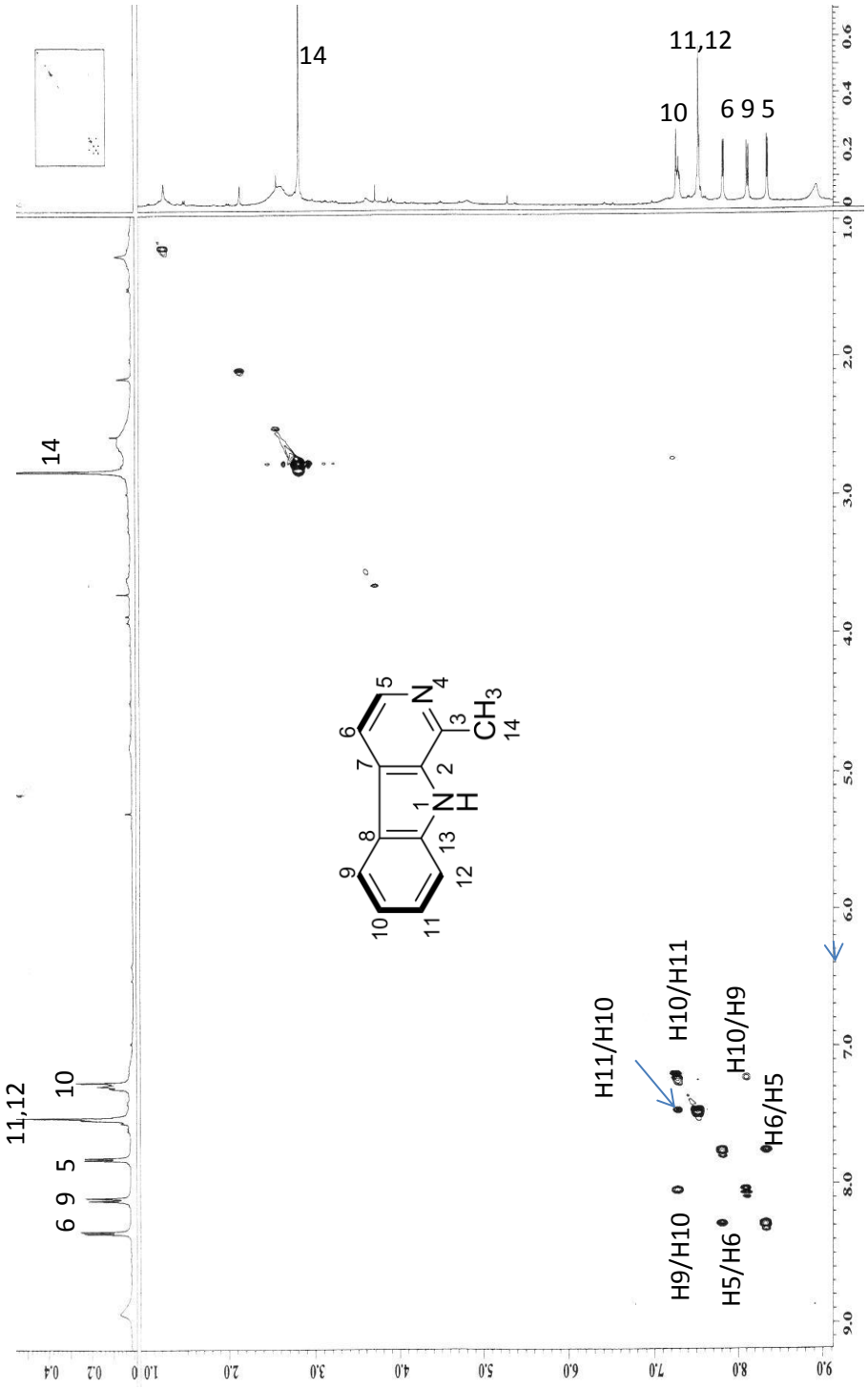


Figure 3.4: COSY Spectrum of Compound **A**

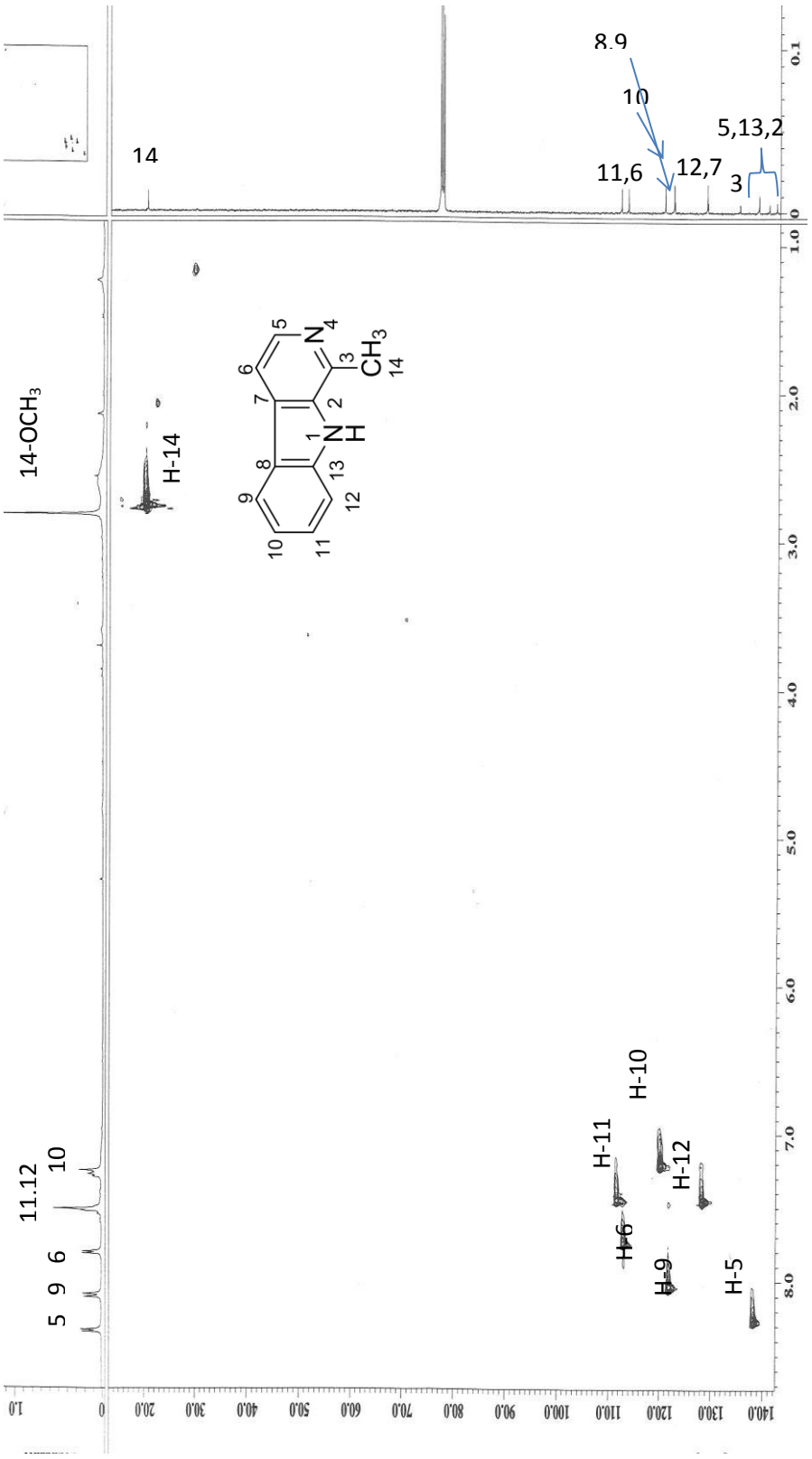


Figure 3.5: HMQC Spectrum of Compound **A**

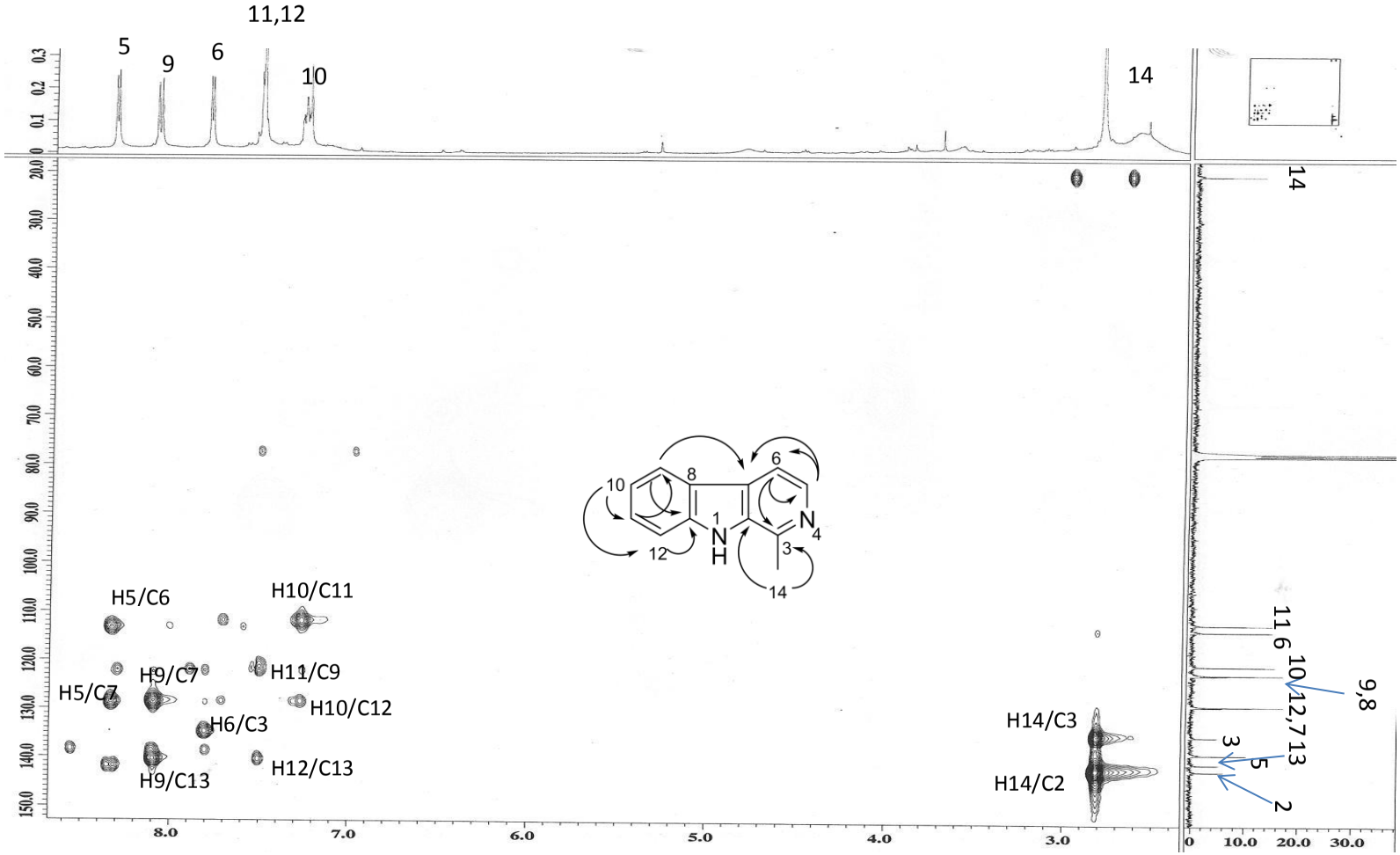
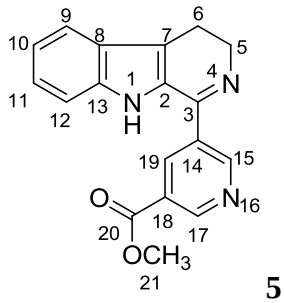


Figure 3.6: HMBC Spectrum of Compound **A**

3.1.2 Compound B: Naucledine 5



5

Compound **B** was yielded as a yellow amorphous solid. A molecular formula was determined to be $C_{18}H_{15}N_3O_2$ by LCMS-IT-TOFF spectrum (Figure 3.7) at *m/z* 306.1213, $[M+H]^+$ with thirteen degrees of unsaturation (four ring and nine double bonds). The UV spectrum exhibited maxima were observed at 330 and 218 nm, suggesting of an indole chromophore.[57-59] The IR spectrum (Figure 3.14) of this alkaloid showed absorption band at 3402 cm^{-1} which indicating the presence of N-H group stretching vibration of an indole alkaloid. The band absorption of carbonyl group (C=O stretching) appeared at 1731 cm^{-1}.

The aromatic region in the ^1H NMR spectrum (Figure 3.8) showed four vicinal aromatic hydrogen signals at δ 7.42 (*d, J*= 8.3 Hz, H-12), 7.68 (*d, J*= 8.3 Hz, H-9), 7.33 (*dd, J*=7.8, 8.3 Hz, H-11), δ 7.23 (*dd, J*=7.8, 8.3 Hz, H-10), suggesting the present of an unsubtituted indole moiety. Methylene peaks were observed at δ 4.12 (*t, J*=6.8 Hz, 2H-5) and δ 3.03 (*t, J*=6.8 Hz, 2H-6), extended of the dihydro β-carboline skeleton.[22] H-5 is more downfield due to the deshielding effect by the neighboring electronegative nitrogen atom. Three pyridine proton signals associated with 3, 5-disubstituted pyridine appeared at δ 9.18 (*d, J*=2.0 Hz, H-17), δ 9.30 (*d, J*=2.0 Hz, H-15) and δ 8.68 *(t, J*=2.0 Hz, H-19). N-H absorption was observed at δ 8.35, (*br s*). One signal of methoxyl group revealed as a singlet at δ 3.98 in the spectrum.

In addition, the COSY spectrum (Figure 3.10) showed cross peaks between; 2H-5 (δ 4.13) /2H-6 (δ 3.03); H-9 (δ 7.68) /H-10 (δ 7.23); H-10 (δ 7.23) /H-11 (δ 7.33); H-11 (δ 7.33) /H-12 (δ 7.42).

The ^{13}C NMR and DEPT-135 spectra (Figure 3.9 and Figure 3.13) were in agreement with the molecular formula deduced from the mass spectrum, accounting for all 18 carbons; seven quaternary carbons, seven sp^2 methines, two sp^3 methylenes, one methyl and one carbonyl carbon. Furthermore, HMBC spectrum revealed a correlation of methoxyl group to the carbonyl carbon at δ 165.5 (C-2), indicating that OMe attached to C-20. Other significant correlations were showed in Figure 3.12. The analysis of the HMBC and HMQC (Figure 3.12 and 3.11) spectra allowed the complete assignment of the ^{1}H and ^{13}C NMR that summarized at Table 3.2.

The analysis of the accumulated data and comparison with literature values [23, 22, 60] that confirmed compound **B** is naucledine **5** previously isolated from *Nauclea diderichii*.

Table 3.2: ^{1}HNMR, ^{13}CNMR (δppm) spectral data of Compound **B** in $CDCl_3$

Position	^{1}H (δ_H, $CDCl_3$) Hz	^{13}C (δ_C, $CDCl_3$)
N-H	8.35 *s*	
2	-	126.3
3	-	156.3
5	4.13 *t J*= 6.8	49.3
6	3.03 *t J*= 6.8	19.3
7	-	
8	-	125.4
9	7.68 *d J*= 8.3	120.3
10	7.23 *dd J*=7.8, 8.3	120.9
11	7.33 *dd J*=7.8, 8.3	125.5
12	7.42 *d J*= 8.3	112.4
13	-	136.9
14	-	125.4
15	9.30 *d J*=2.0	151.8
17	9.18 *d J*=2.0	152.6
18	-	133.3
19	8.68 *t J*=2.0	136.5
20		165.5
21	3.98 *s*	52.8

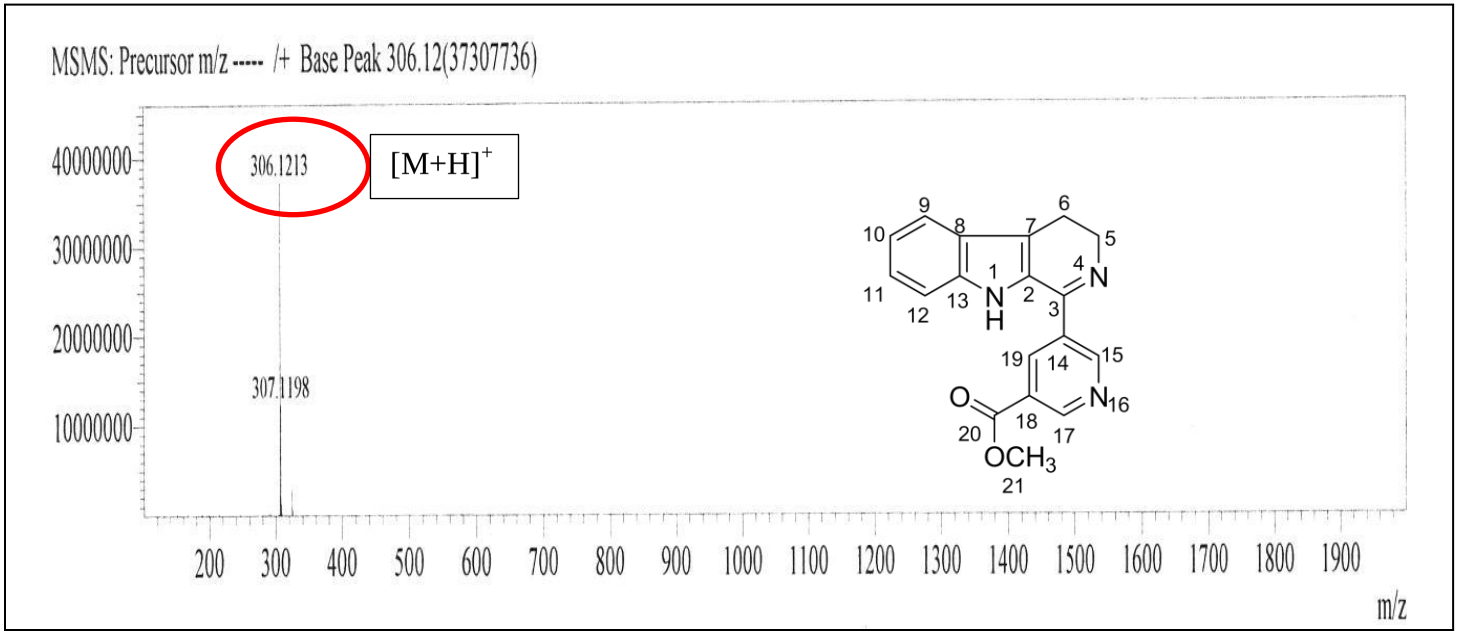


Figure 3.7: LCMS-IT-TOFF Spectrum of Compound **B**

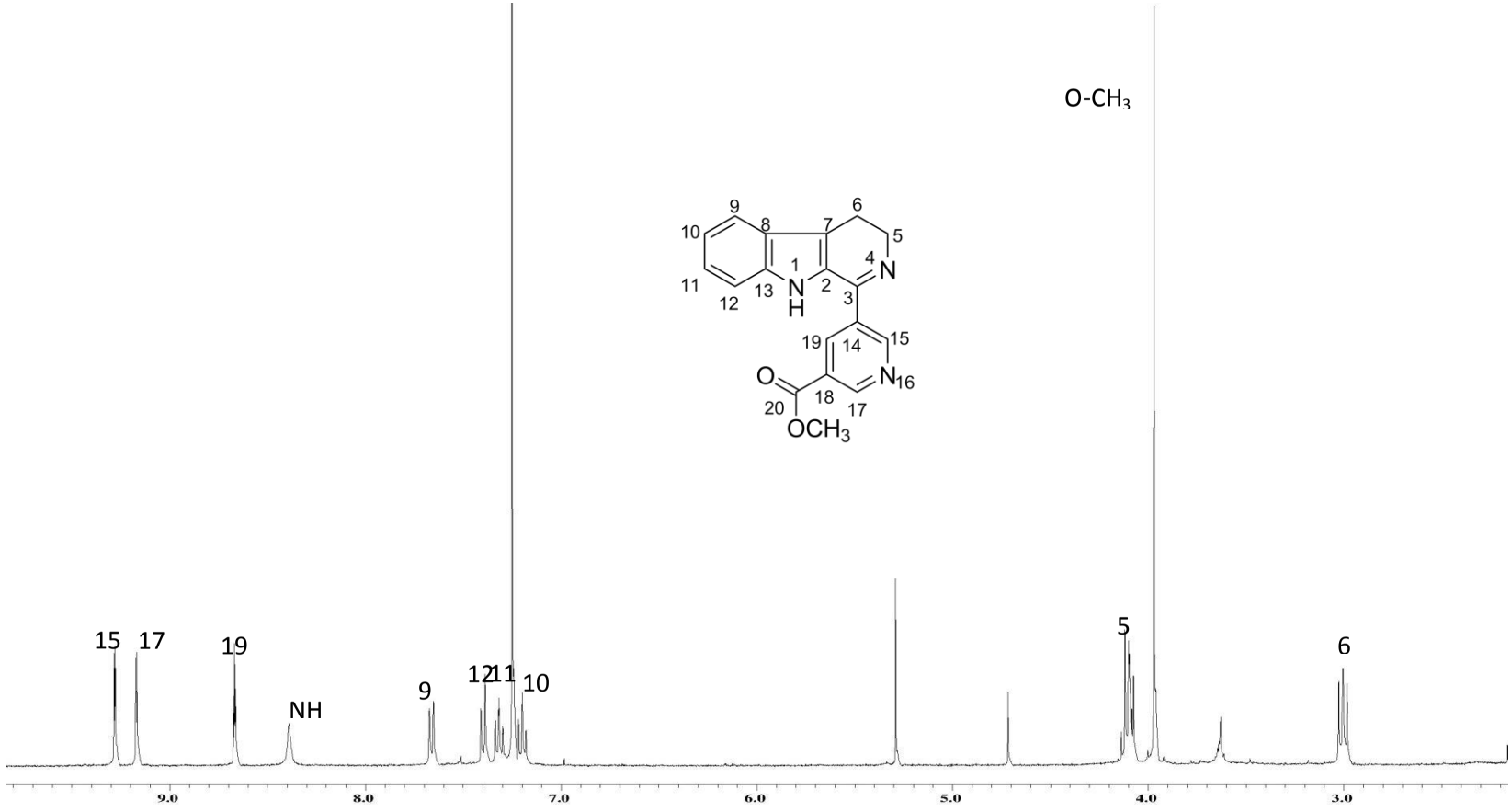


Figure 3.8: [1]HNMR Spectrum of Compound **B**

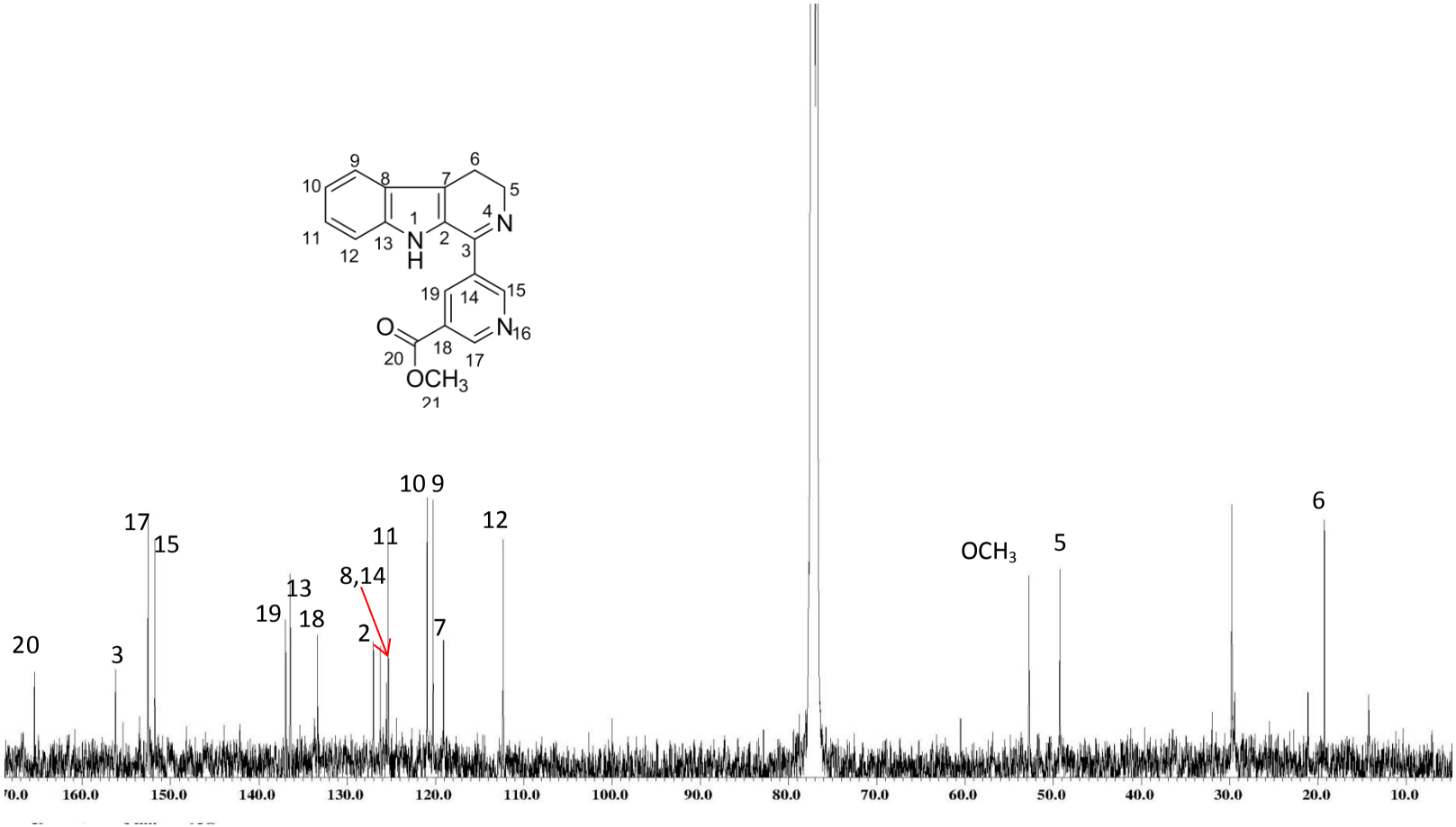


Figure 3.9: ^{13}CNMR Spectrum of Compound **B**

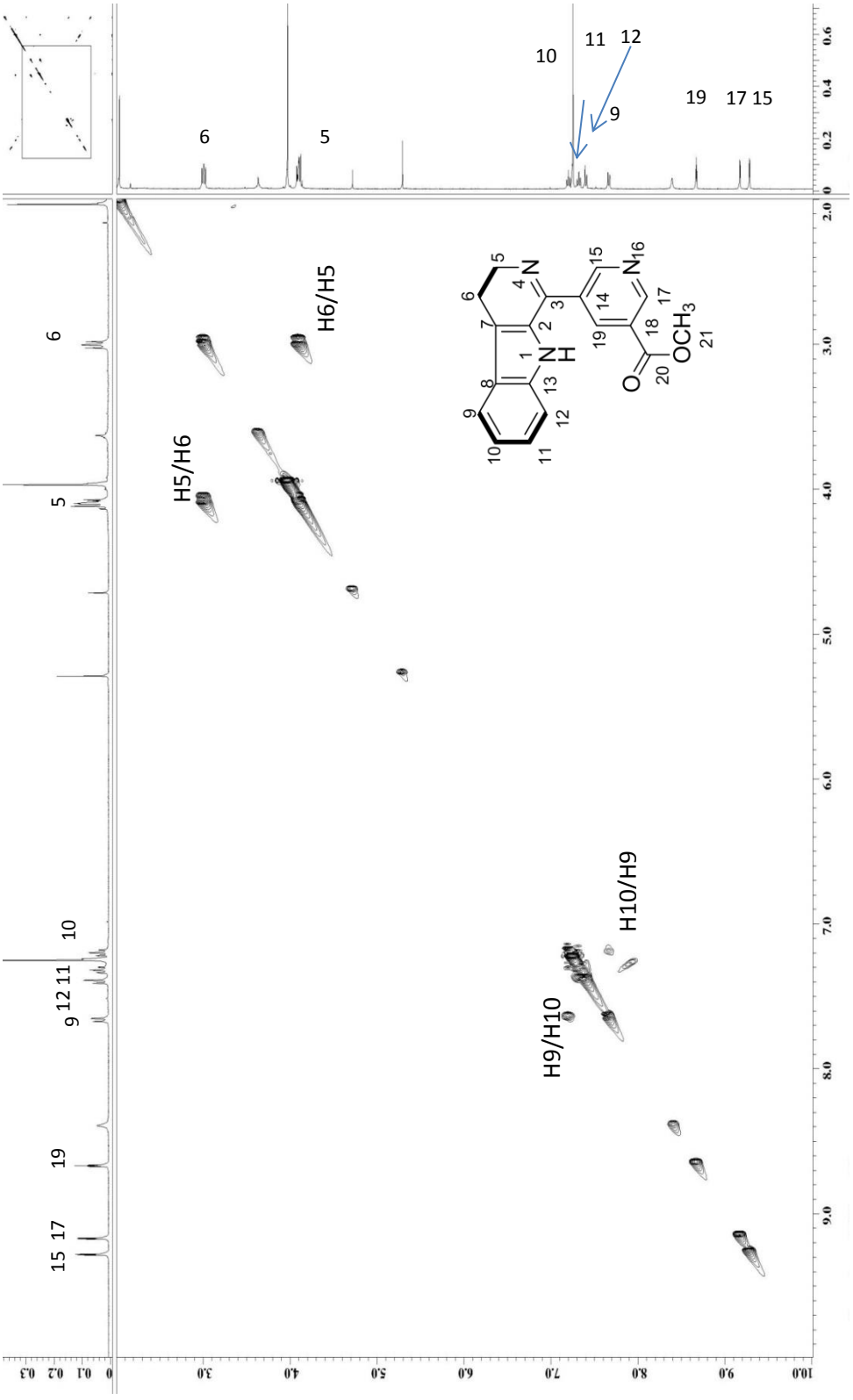


Figure 3.10: COSY Spectrum of Compound **B**

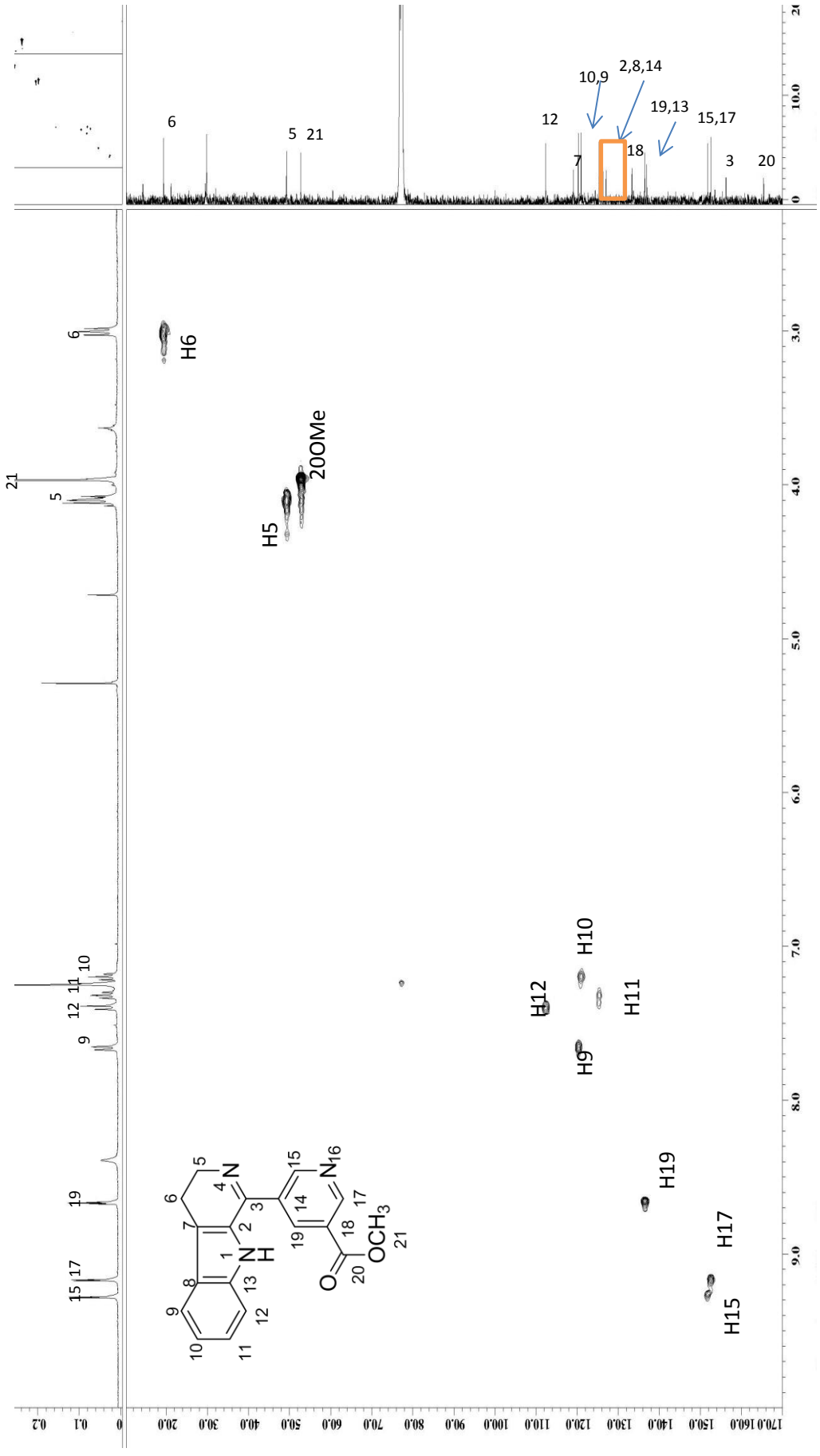


Figure 3.11: HMQC Spectrum of Compound **B**

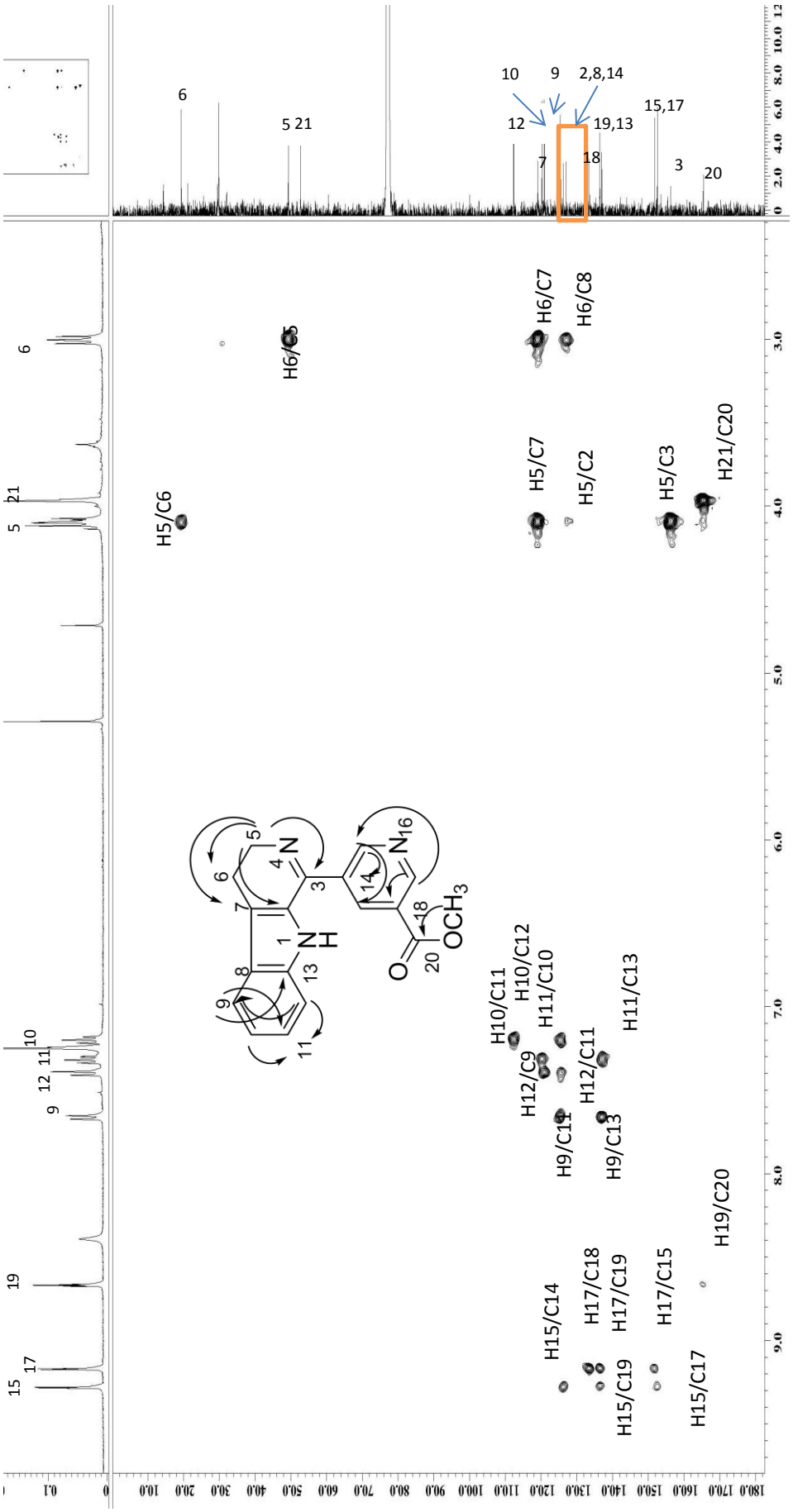


Figure 3.12: HMBC Spectrum of Compound **B**

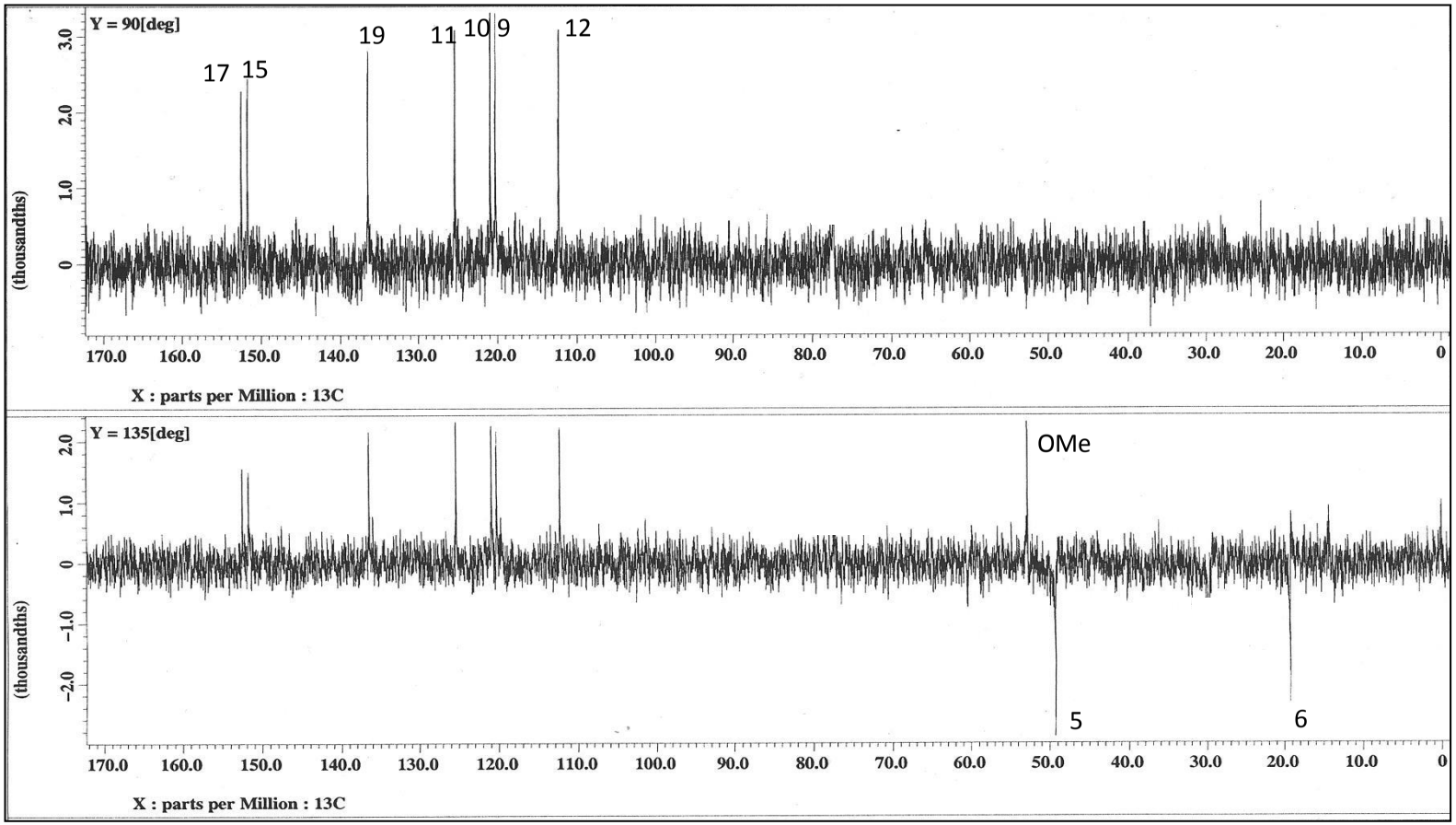


Figure 3.13: DEPT Spectrum of Compound **B**

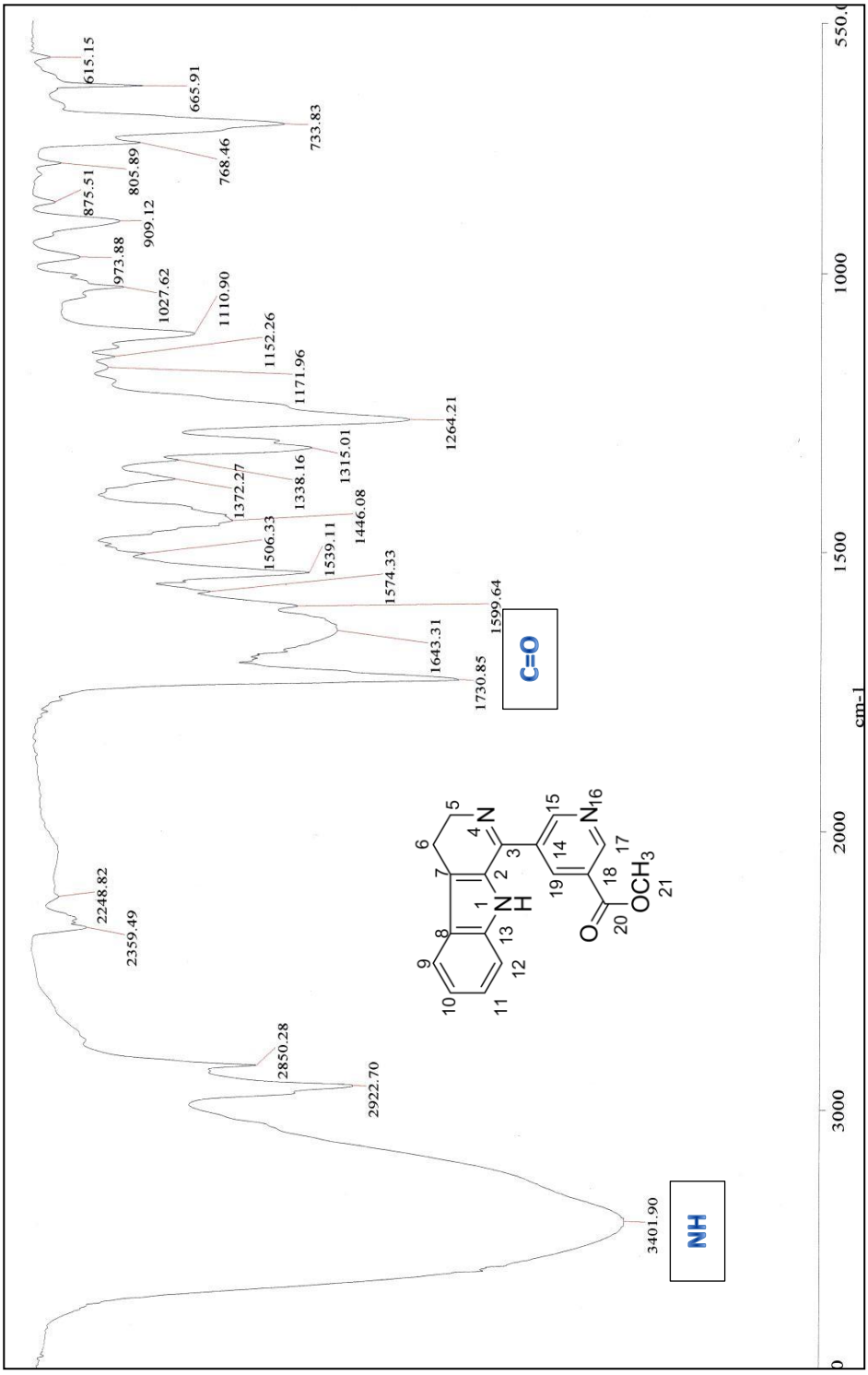
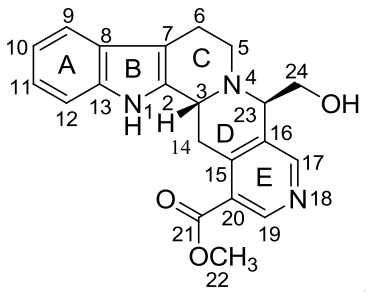


Figure 3.14: IR Spectrum of Compound **B**

3.1.3 Compound C: Cadamine 42



42

Compound **C** was afforded as an orange-red amorphous. The IR spectrum (Figure 3.24) exhibited a broad absorption at 3306 cm^{-1} due to the existing of NH/OH group while band absorption at 1727 cm^{-1} was assigned to a carbonyl group. The UV displayed maxima typical of an indole chromophore [57-59] at 324 and 222 nm. The LCMS-IT-TOFF spectrum (Figure 3.16) for compound **C** showed a molecular ion peak at *m/z* 364.1638, $[M+H]^+$ corresponding to the molecular formula $C_{21}H_{21}N_3O_3$ with thirteen degrees of unsaturation.

Analysis of ^{1}H NMR spectrum (Figure 3.17) revealed four aromatic proton signals of the β-carboline moiety at δ 7.48(*d, J*=8.0), 7.10(*dd, J*=8.0, 8.0), 7.16(*dd, J*=8.0, 8.0), and 7.27(*d, J*=8.0) assignable to H-9, H-10, H-11 and H-12, respectively. The broad singlet signal of N-H was observed at δ 7.95. Six multiplet peaks appeared at δ 3.22 (H-5), 2.94 (H-5'), 2.80 (H-6), 2.98 (H-6'), 3.25 (H-14) and 4.43 (H-3) respectively. The methylene signals H-24 were observed more downfield at δ 3.61 (H-24) and 3.75 (H-24') because of electron withdrawing effect of the adjacent from hydroxyl group. Two methine protons peaks of chiral carbons exhibited as multiplet at δ 4.43 (H-3) and δ 4.10 (H-23). At ring E two singlet pyridine protons resonate at δ 8.51(H-17) and 8.96 (H-19). In addition, the singlet signal at δ 3.88 characterizing a methoxyl group was also observed in ^{1}H NMR spectrum.

The assignment of all the vicinal protons of the compound **C** were confirmed by the analyzing the COSY spectrum (Figure 3.19). The cross peaks showed the correlations between H-6 (2.80) / H-5 (2.94), H-6 (2.80)/ H-5'(3.22), H-3 (4.43) / H-14 (3.25), H-3(4.43) / H-14'(3.51), H-9 (7.48) / H-10 (7.10), H-10 (7.10) / H-11(7.16), H-11(7.16) / H-12 (7.27), H-17 (8.51) / H-23(4.10), and H-24 (3.61)/ H-23 (4.10) .

The ^{13}C NMR and DEPT spectra (Figure 3.18 and Figure 3.22) were in agreement with the molecular formula implied from the mass spectrum, indicating for all 21 carbons signals; seven quaternary carbons, seven methine carbons, four methylene carbons, one carboxyl carbon, one methyl carbon and one carbonyl carbon. One of the peak appeared at upfield region at δ 21.9 which belong to one of the methylene groups (C-6). Carbonyl carbon appeared at very downfield region at δ 166.33 in ^{13}C NMR spectrum.

In addition, the relative stereochemistry at C-3 and C-23 were assigned by examining the NOESY spectrum (Figure 3.23) showed cross-peaks between H-3 (δ 4.43)/H-14 (δ 3.25), H-3/H-5' (δ 3.22), H-3/H-24 (δ3.61), H-17 (δ 8.51)/H-23 (δ 4.10), and H-17 (δ 8.51)/H-24' (δ3.75), respectively. Therefore the relative stereochemistry of compound C is depicted in Figure 3.15.

Complete assignments of ^{1}H-NMR, ^{13}C-NMR, HMBC and NOESY data of compound **C** were summarized in Table 3.3. From the analysis of all spectral data and comparison with literature values[61] suggested that compound **C** is known indole cadamine **42**.

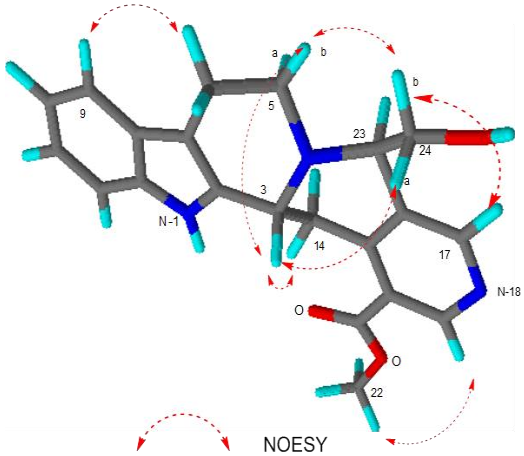


Figure 3.15: Selected NOESY Correlation of Compound **C**

Table 3.3: ^{1}HNMR, ^{13}CNMR (δppm), HMBC and NOESY spectral data of Compound **C** in $CDCl_3$

Position	^{1}H (δ_H, $CDCl_3$)	^{13}C (δ_C, $CDCl_3$)	HMBC	NOESY
N-H	7.95 *s*	-	2, 8, 7	
2	-	134.9		
3	4.43 *m*	46.3	2	14, 5', 24
5	2.94 *m*	47.6	3	
5'	3.22 *m*		2, 6, 7, 23	
6	2.80 *m*	21.9	7	
6'	2.98 *m*		5, 2	
7	-	107.9		
8	-	126.9		
9	7.48 *d* *J*=8.0	118.3		
10	7.10 *dd* *J*=8.0, 8.0	119.4	8, 12	
11	7.16 *dd* *J*=8.0, 8.0	121.9	9, 13	
12	7.27 *d* *J*=8.0	111.1	8, 10	
13	-	136.2		
14	3.25 *m*	28.3	15, 16, 20	
14'	3.51 *m*		2, 3, 13	
15	-	124.5		
16	-	130.8		
17	8.51 *s*	151.8	16, 19, 23	23, 24'
19	8.96 *s*	150.0	15, 20, 21	
20	-	144.8		
21	-	166.3		
22-OMe	3.88 *s*	52.4	20, 21	
23	4.10 *m*	62.6	5, 16, 24	
24	3.61 *m*	63.6	23	
24'	3.75 *m*			

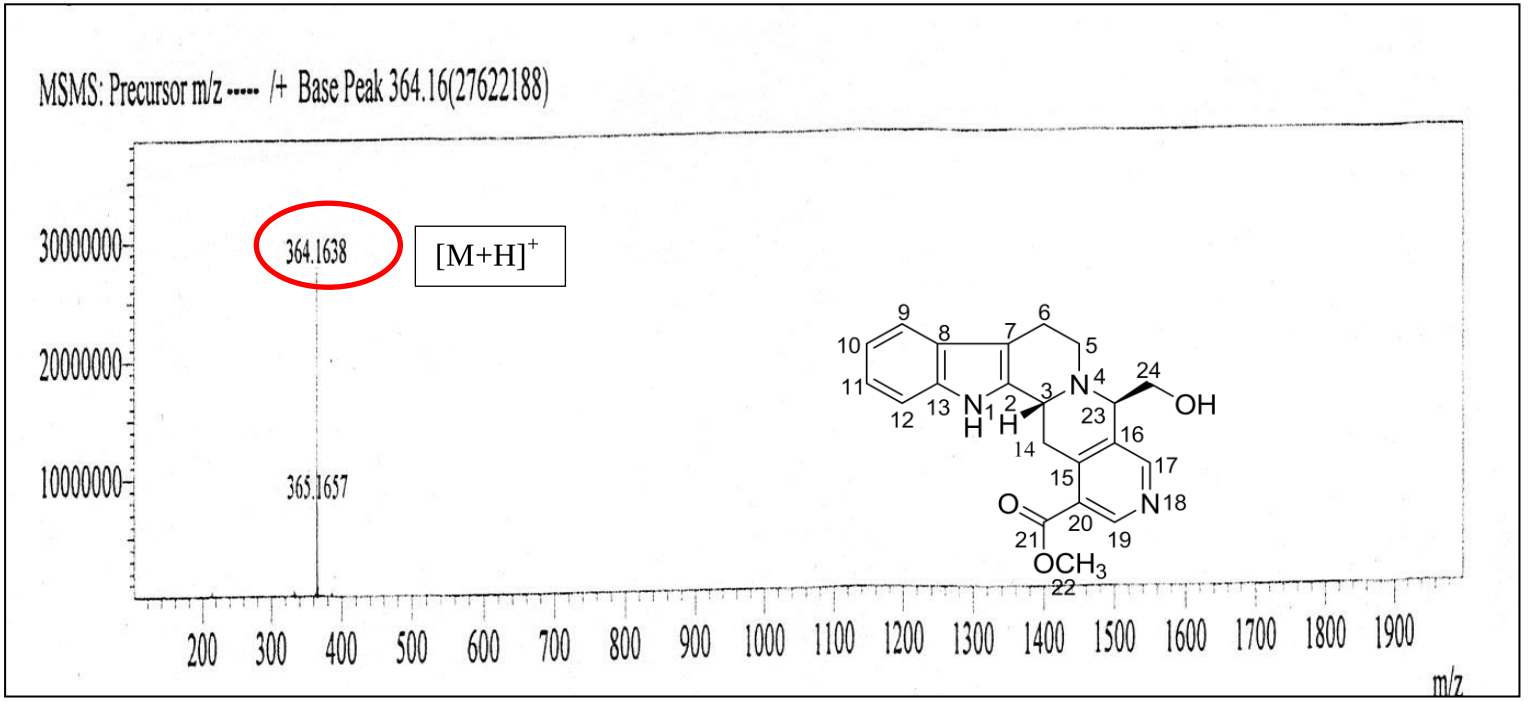


Figure 3.16: LCMS Spectrum of Compound **C**

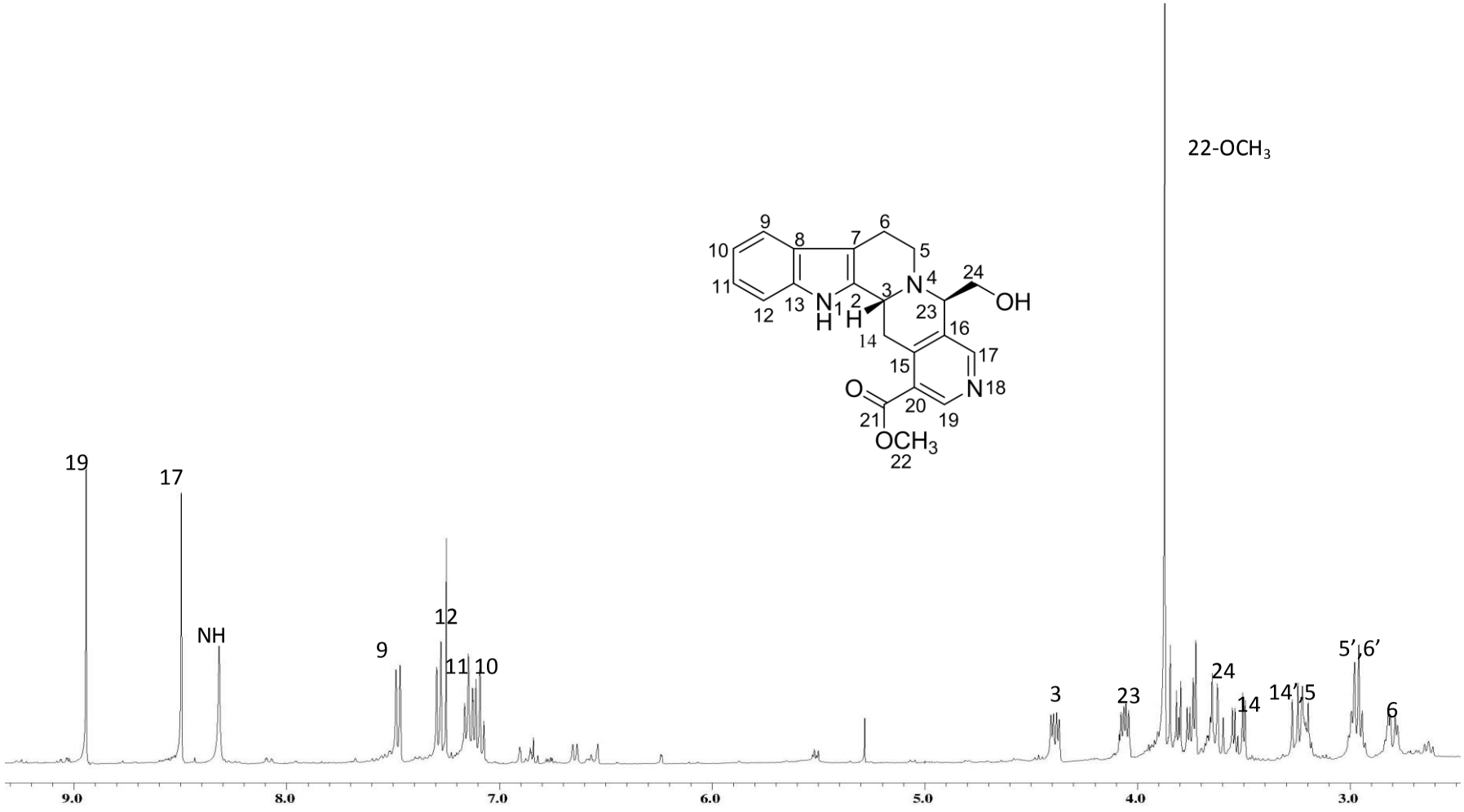


Figure 3.17: ^{1}HNMR Spectrum of Compound **C**

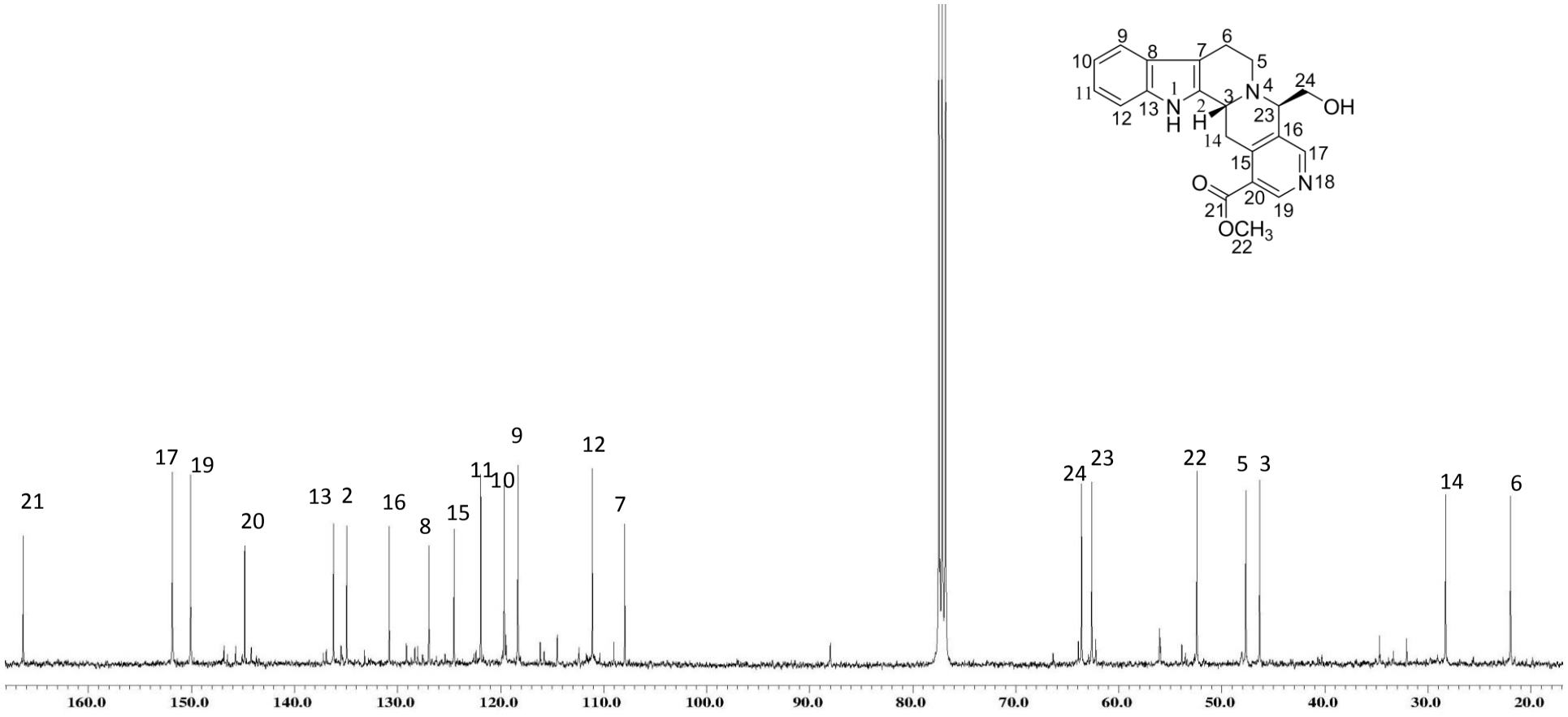


Figure 3.18: ^{13}CNMR Spectrum of Compound **C**

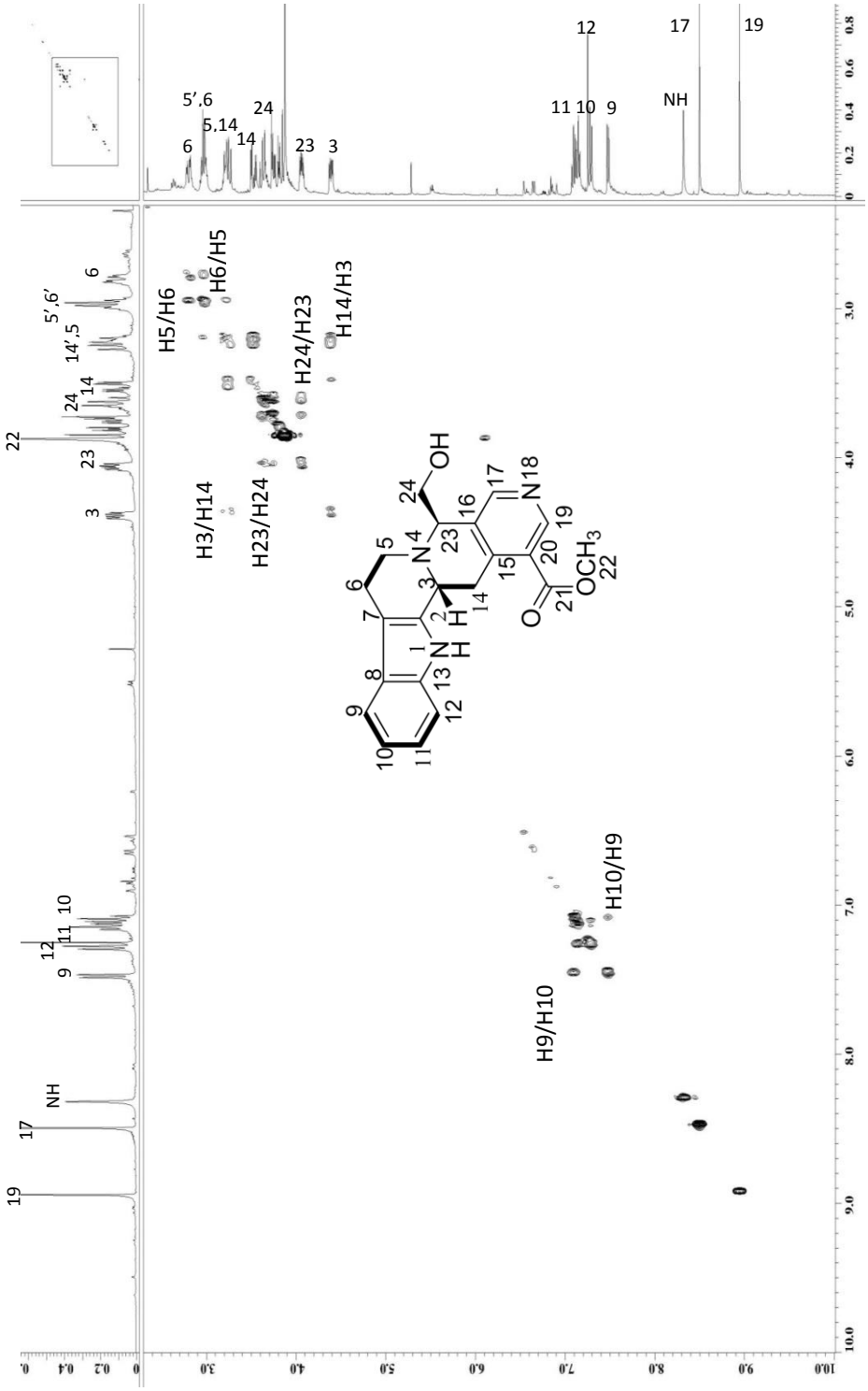


Figure 3.19: COSY Spectrum of Compound **C**

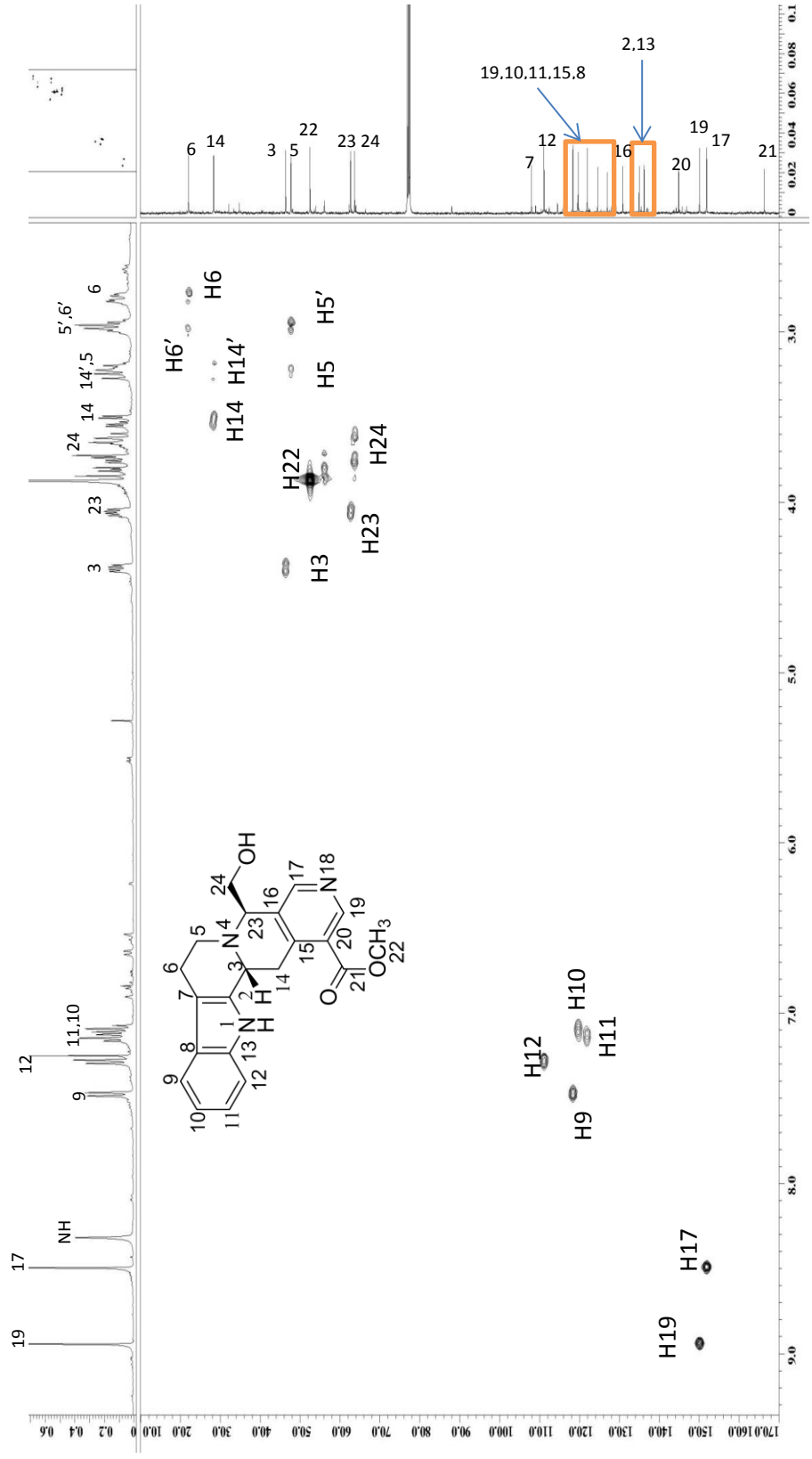


Figure 3.20: HMQC Spectrum of Compound **C**

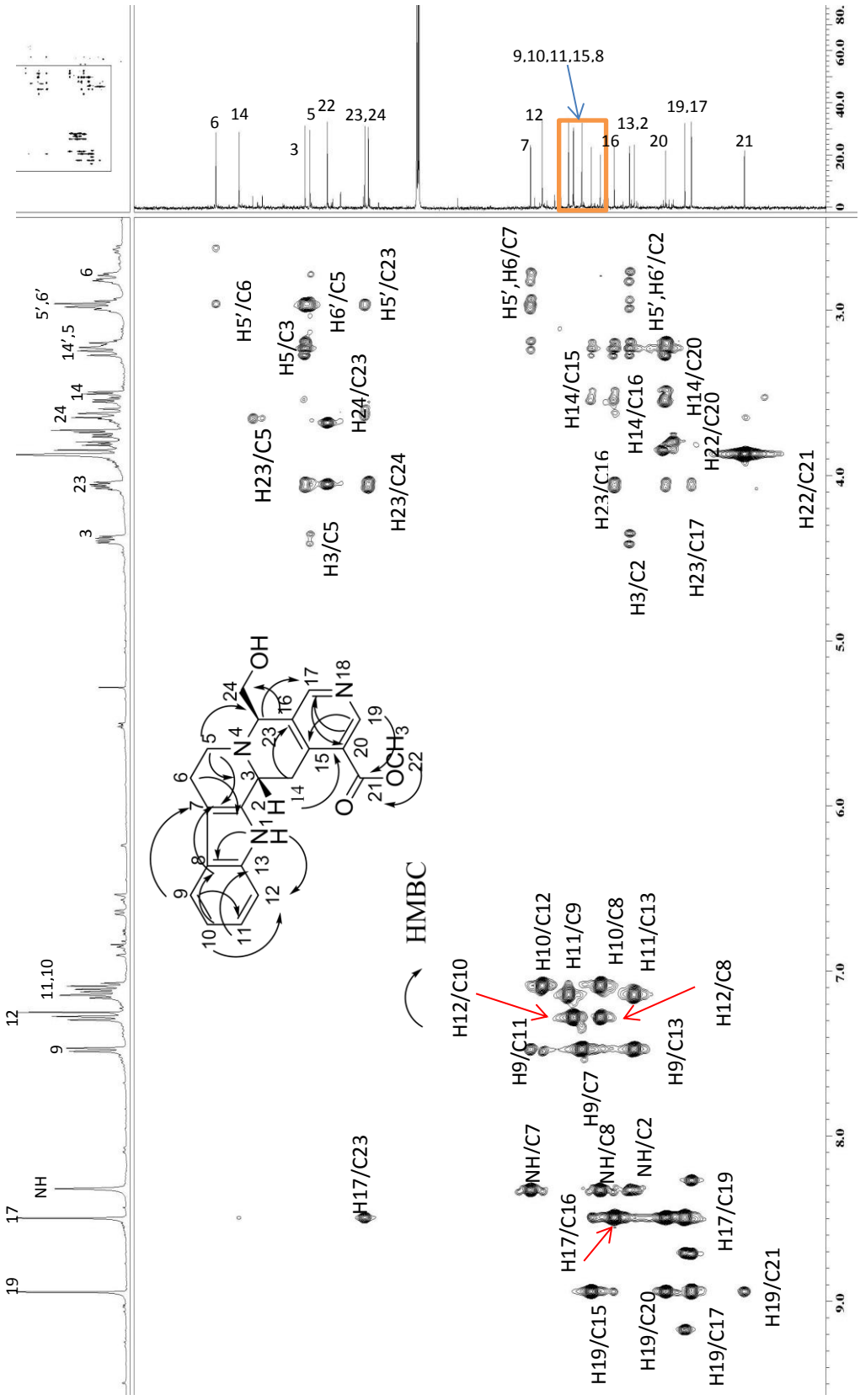


Figure 3.21: HMBC spectrum of Compound **C**

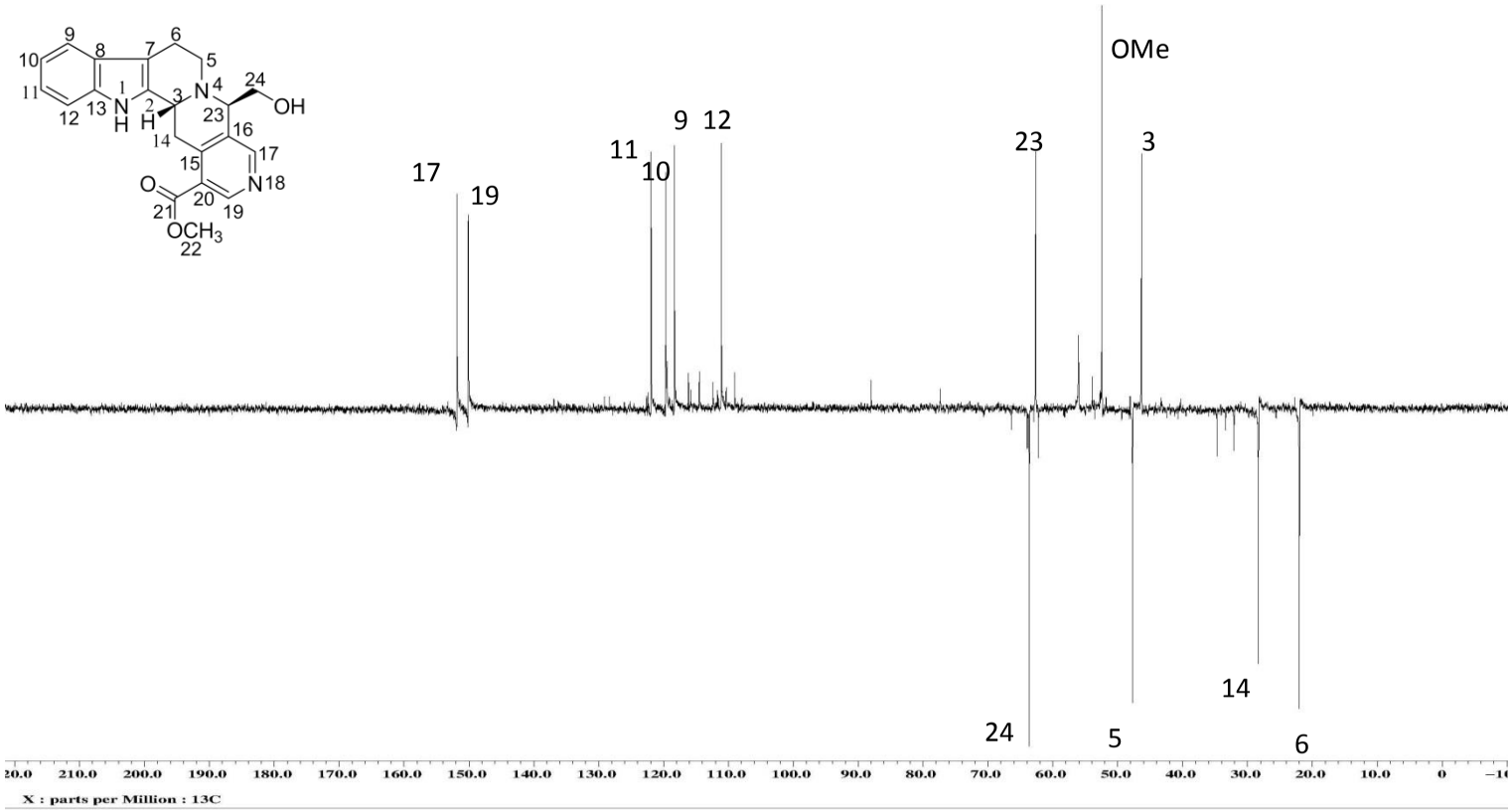


Figure 3.22: DEPT-135 Spectrum of Compound **C**

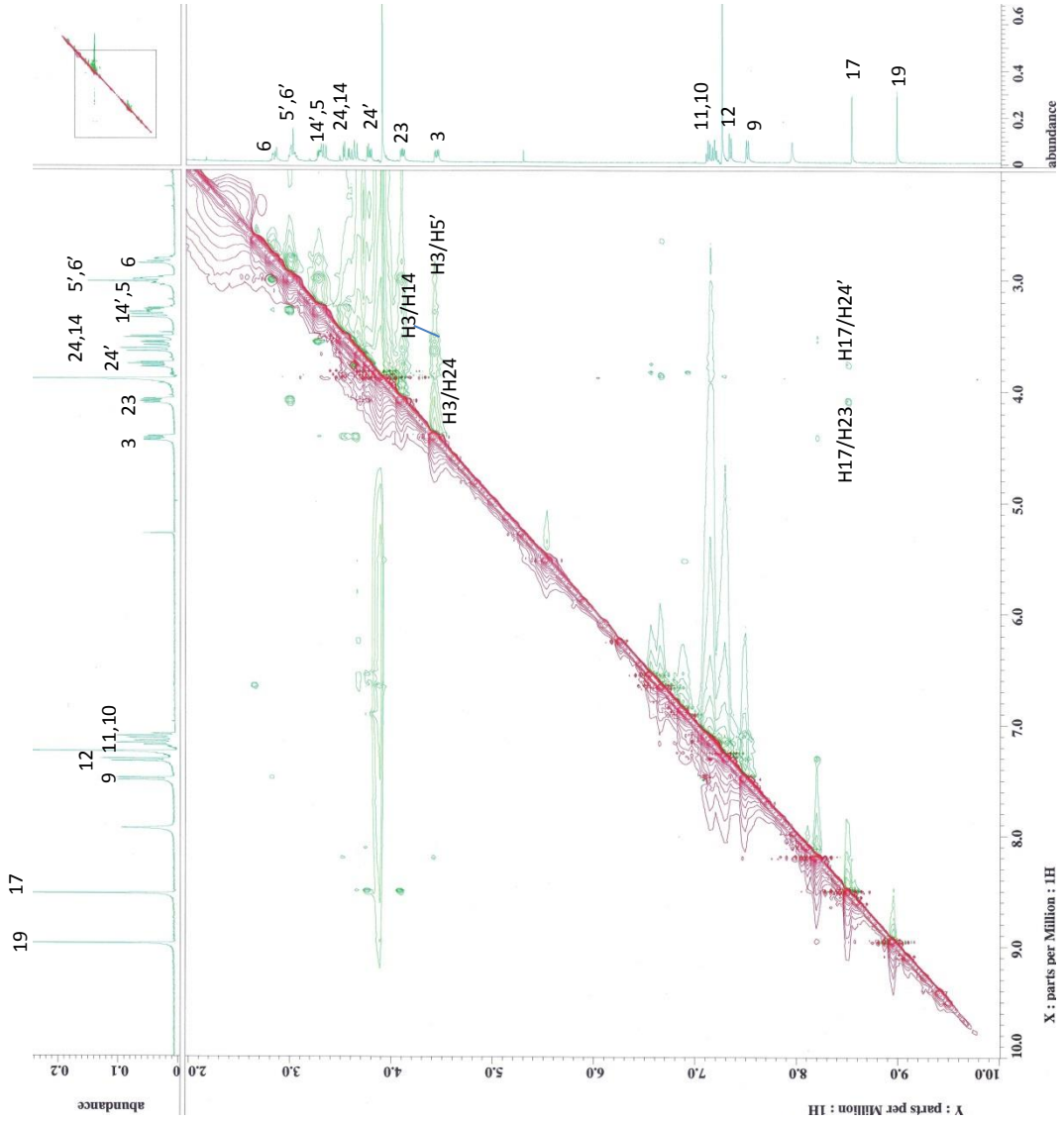


Figure 3.23: NOESY Spectrum of Compound **C**

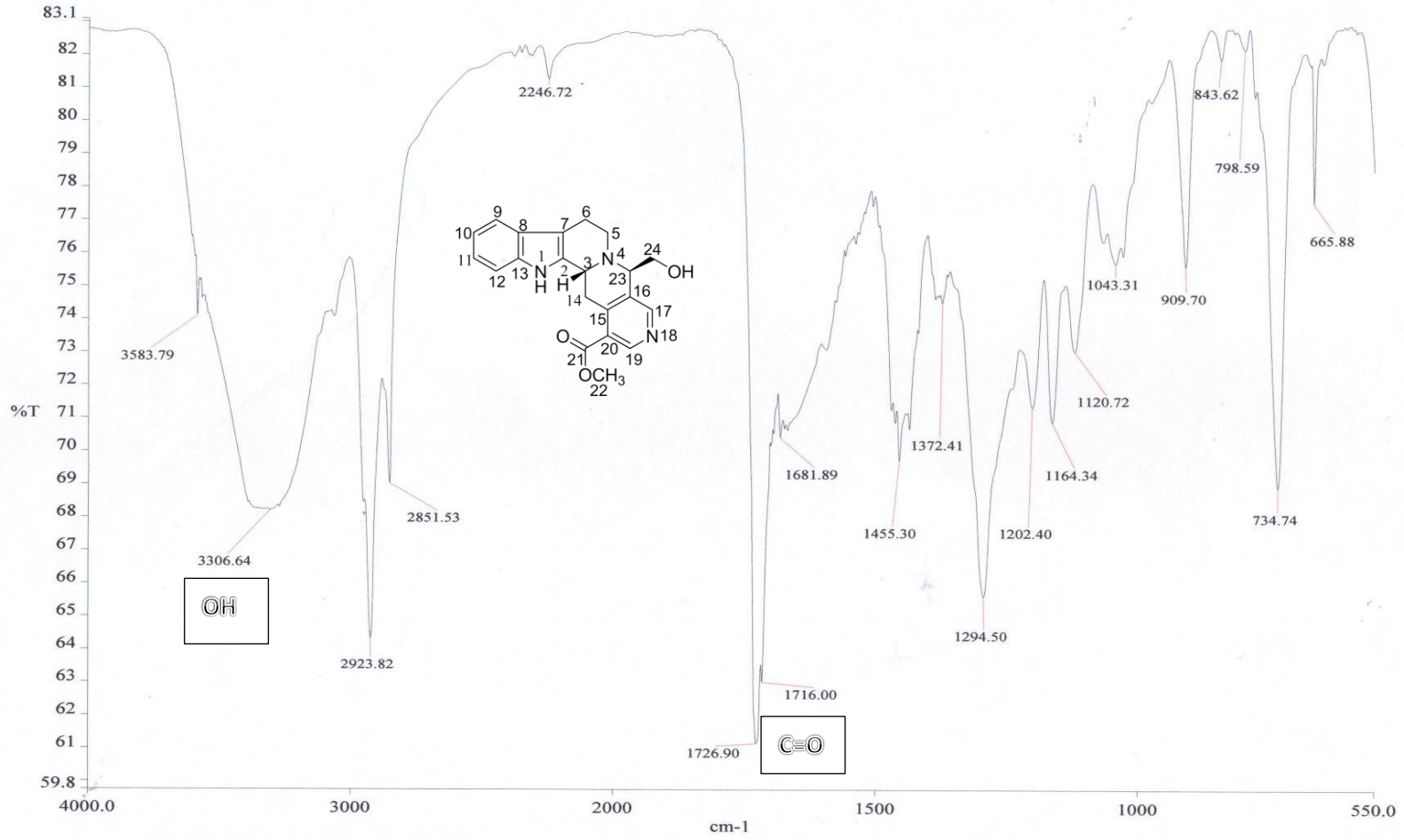
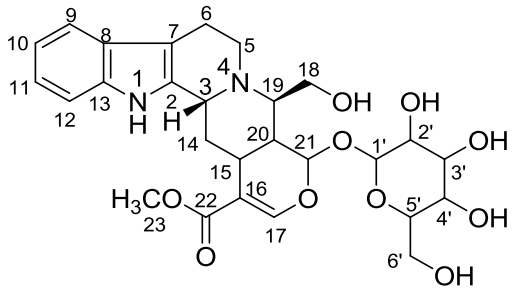


Figure 3.24: IR Spectrum of Compound **C**

3.1.4 Compound D: Isodihydrocadambine 78



78

Compound **D** was obtained as a brown amorphous solid. The LCMS-IT-TOFF spectrum (Figure 3.25) gave a very significant molecular ion peak at *m/z* 547.2335 $[M+H]^+$ which agreement to the molecular formula $C_{27}H_{34}N_2O_{10,}$ with twelve degrees of unsaturation. The UV maxima were observed at 275, 271 and 220 nm which revealed of an chromophore.[57-59] The IR spectrum (Figure 3.31) of this alkaloid showed absorption bands at 3369 and 1692 cm^{-1} indicated the presence of a primary hydroxyl and conjugated carbonyl group.

Further analysis on the ^{1}HNMR spectrum (Figure 3.26) showed two of four aromatic proton signals appeared as doublets at δ 7.38 (*J*=7.8 Hz) and 7.25 (*J*=8.2 Hz), and the others as two were triplets at δ 7.07 (*J*=7.3 Hz) and 7.01 (*J*=7.3 Hz) attributed to H-9, H-12, H-11 and H-10, respectively. The characteristic downfield signal of H-17 at δ 7.47 suggested that compound D has a pyridine skeleton. The methylene proton signals appeared as multiplet at 2.95-2.99 (H-5a), 3.09-3.14 (H-5b), 2.68 (H-6a) and 2.82-2.99 (H-6b) respectively.[62] One methoxy signal which attached to a carbonyl carbon was observed at δ 3.37.

In addition, the COSY spectrum (Figure 3.28) showed cross peaks such as H-5a (2.95-2.99,)/ H-6a (2.68), H-9 (7.38)/ H-10 (7.01), H-10 (7.01)/ H-11(7.07), H-11

(7.07)/ H-12 (7.25) ,H-1’(4.75)/ H-21(5.38), H-14a (1.64)/ H-14b (2.16), H-19 (4.24)/ H18a (2.87-2.89).

The ^{13}C NMR spectrum (Figure 3.27) is in agreement with the molecular formula indicated from the mass spectrum, suggesting for all 27 carbons; five quaternaries, ten methines, four methylenes, one carbonyl, one methyl and six glucose carbons, respectively. Carbonyl carbon appeared at very downfield region at δ 168.6 in ^{13}C NMR spectrum. The glucose carbon signals can be seen at the upfield region δ 60.8 to 76.8 (C-2’ to C-5’). The anomeric C-1’ signal resonated more downfield at δ 99.8.

The HMBC spectrum showed the correlations of an acetal proton at 5.38 H-21 (5.38 *d*, *J*= 9.1) and H-5’ (3.20- 3.2, *m*) to C-1’ (99.8) indicating the presence of a glucose unit at C-21 (96.2). Other significant HMBC correlations were shown in Figure 3.30. Table 3.4 showed the complete assignments of all protons, carbons and HMBC.

Based on the observed data and the literature values[63, 62, 13, 64] compound D is confirmed to be isodihydrocadambine **78**.

Table 3.4: ^{1}HNMR, ^{13}CNMR (δppm) and HMBC spectral data of Compound **D** in $CDCl_3$+ CD_3OD

Position	^{1}H (δ_H ,Hz)	^{13}C ($\delta_{C\ ppm}$)	HMBC
2	-	134.4	
3	3.70-3.74 *m*	63.4	
5a	2.95-2.99 *m*	55.0	6,3
5b	3.09-3.14 *m*		
6a	2.68 *m*	22.7	5,7
6b	2.82-2.99 *m*		
7	-	108.2	
8	-	126.8	
9	7.38 *d J*=7.8	118.0	7,11,13
10	7.01 *t J*=7.3	119.3	8,12
11	7.07 *t J*=7.3	121.6	9,13
12	7.25 *d J*=8.2	111.1	8,10
13	-	136.4	
14a	1.64-1.73 *m*	36.5	20
14b	2.16 *d J*=14.2		
15	2.95-2.99 *m*	33.1	
16	-	109.7	
17	7.47 *s*	153.2	22,21,16
18a	2.87-2.89 *m*	58.3	20
18b	3.09-3.14 *m*		
19	4.24 *t J*=5.9	64.5	15
20	1.87-1.92 *m*	43.1	21
21	5.38 *d J*=9.1	96.2	1’
22	-	168.6	
1’	4.75 *d J*=7.8	99.8	21
2’	3.36-3.40 *m*	72.7	1’,3’
3’	3.44-3.51 *m*	76.5	2’,4’
4’	3.44-3.51 *m*	68.9	5’
5’	3.20-3.23 *m*	76.8	
6’	3.69-3.72 *m*	60.8	
23-OCH_3	3.73 s	51.7	22

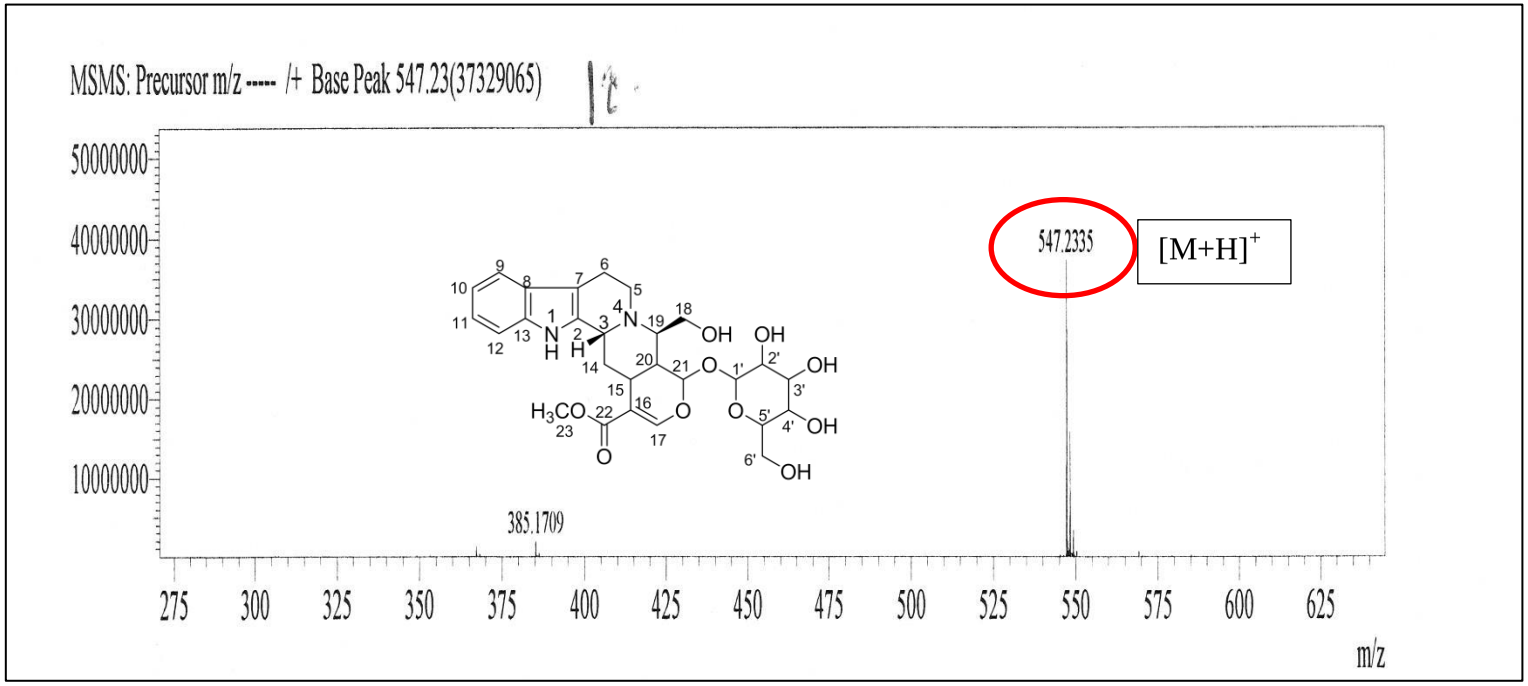


Figure 3.25: LCMS Spectrum of Compound **D**

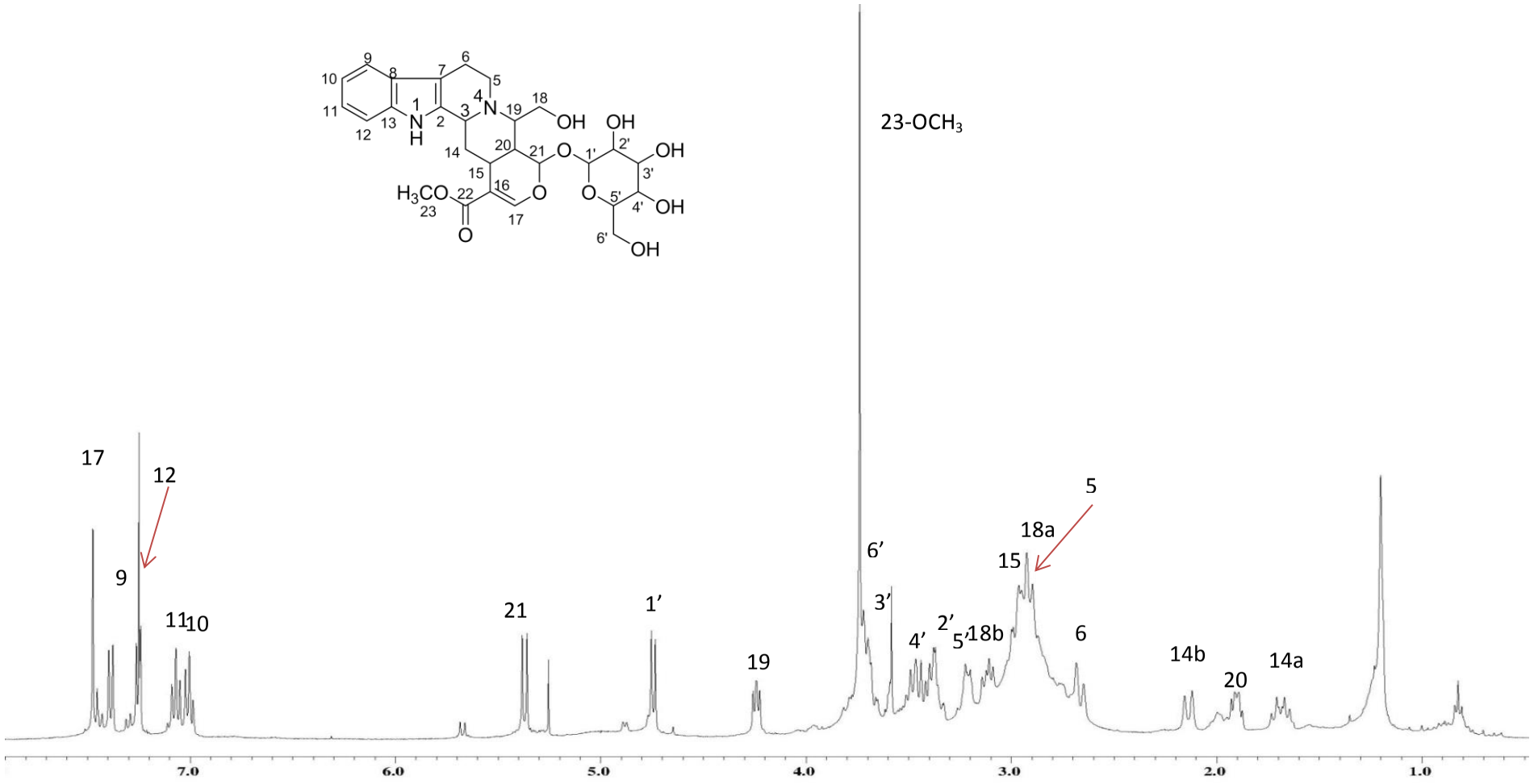


Figure 3.26: [1]HNMR Spectrum of Compound **D**

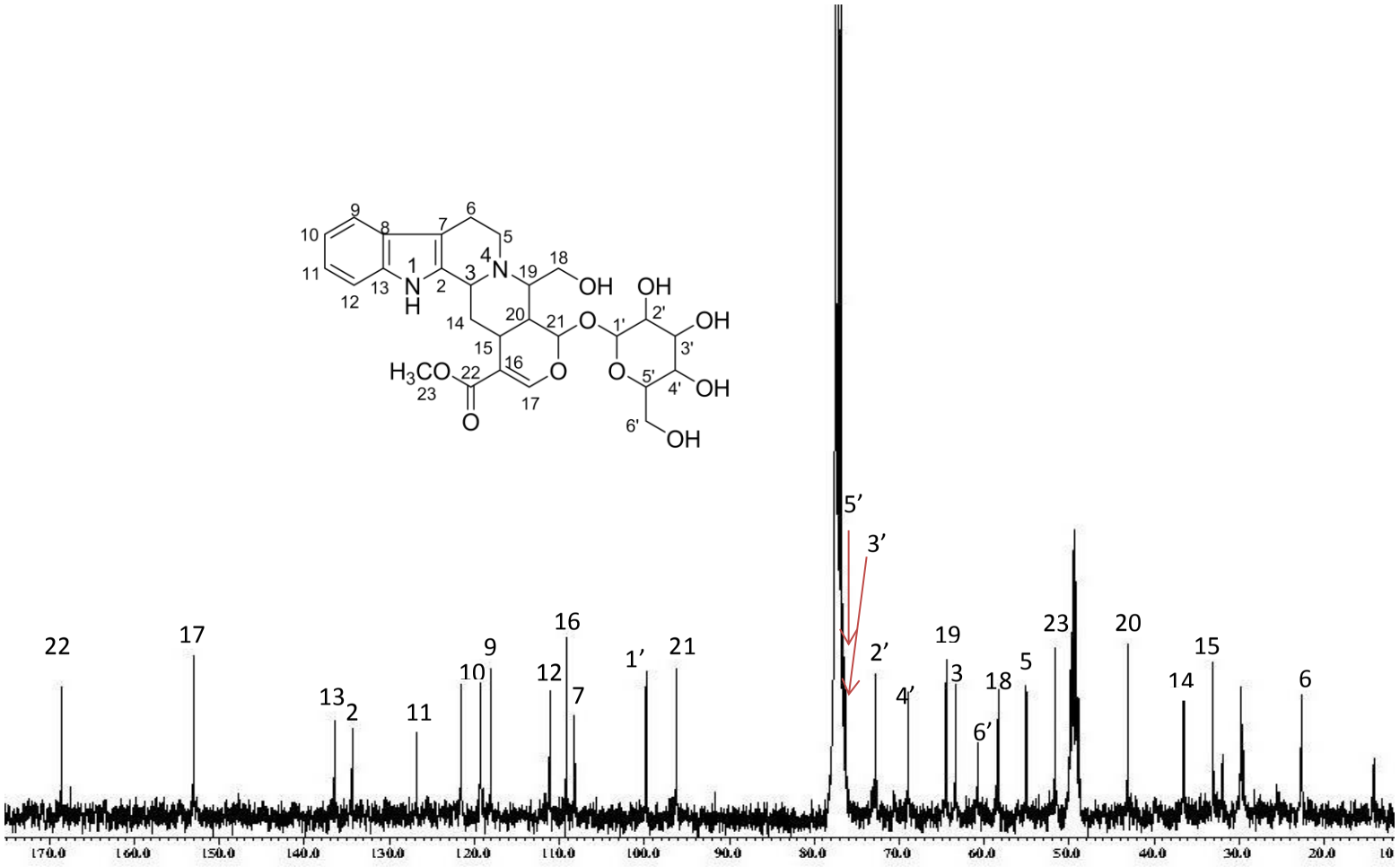


Figure 3.27: ^{13}CNMR Spectrum of Compound **D**

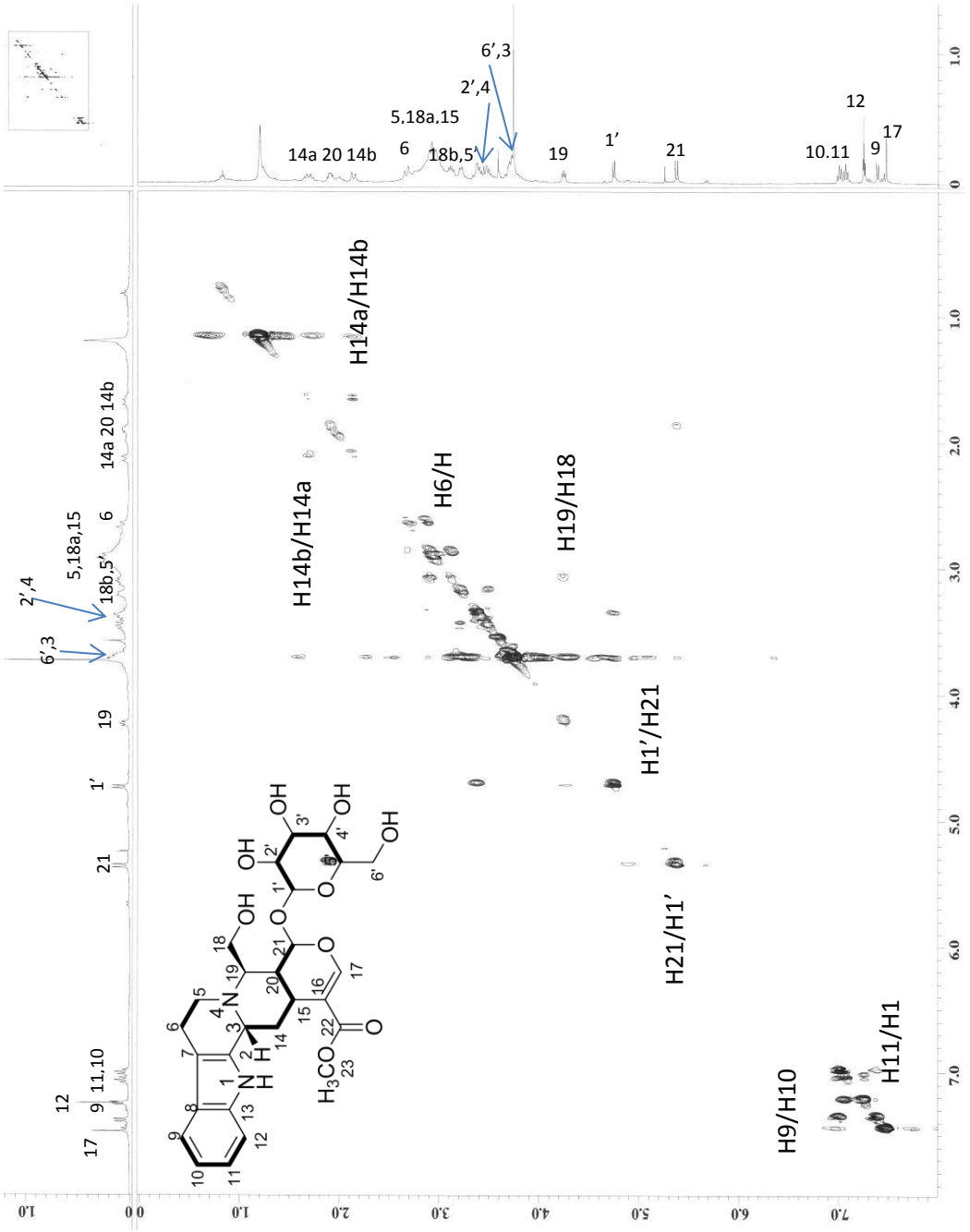


Figure 3.28: COSY spectrum of Compound **D**

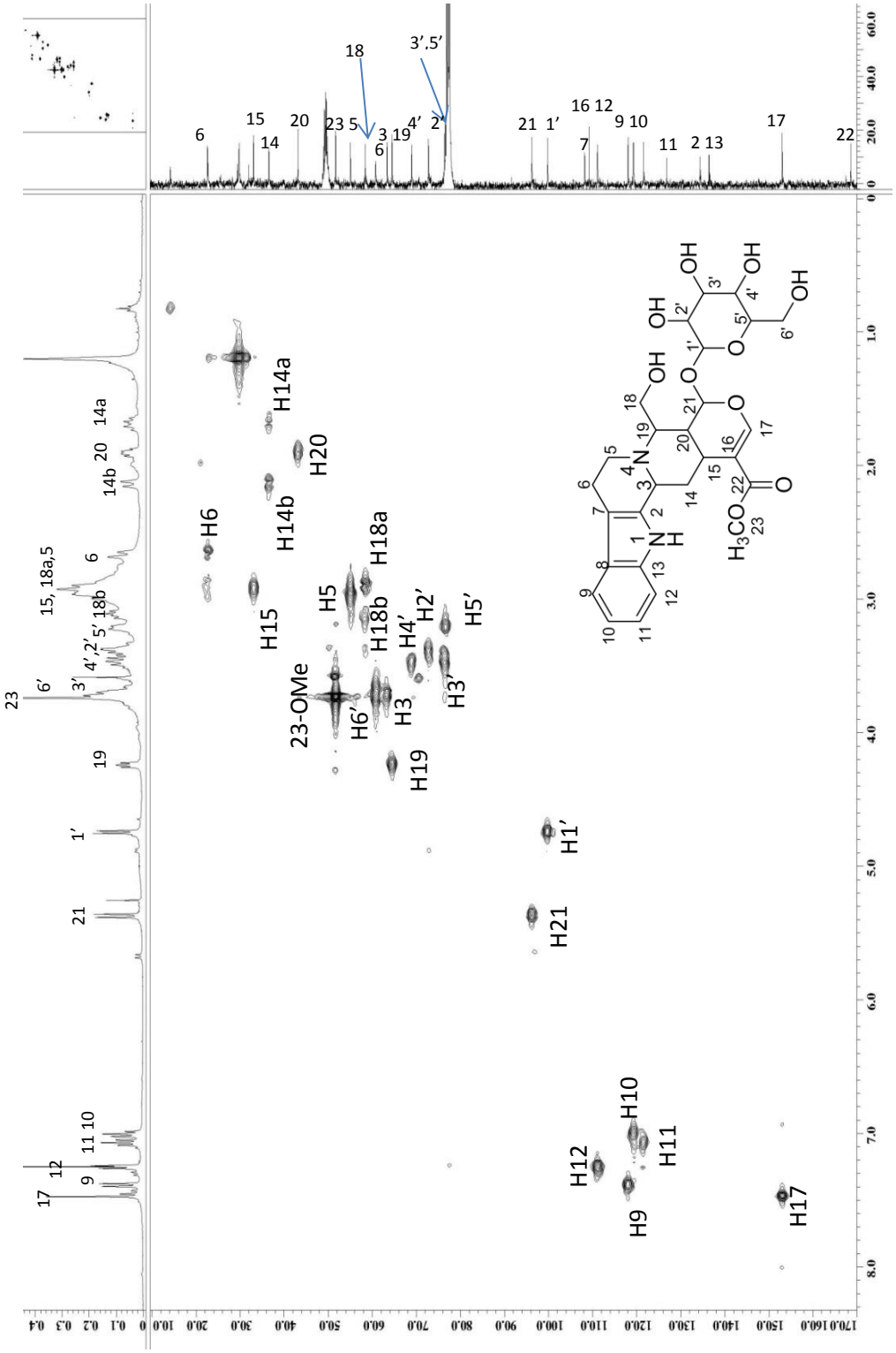


Figure 3.29: HMQC Spectrum of Compound **D**

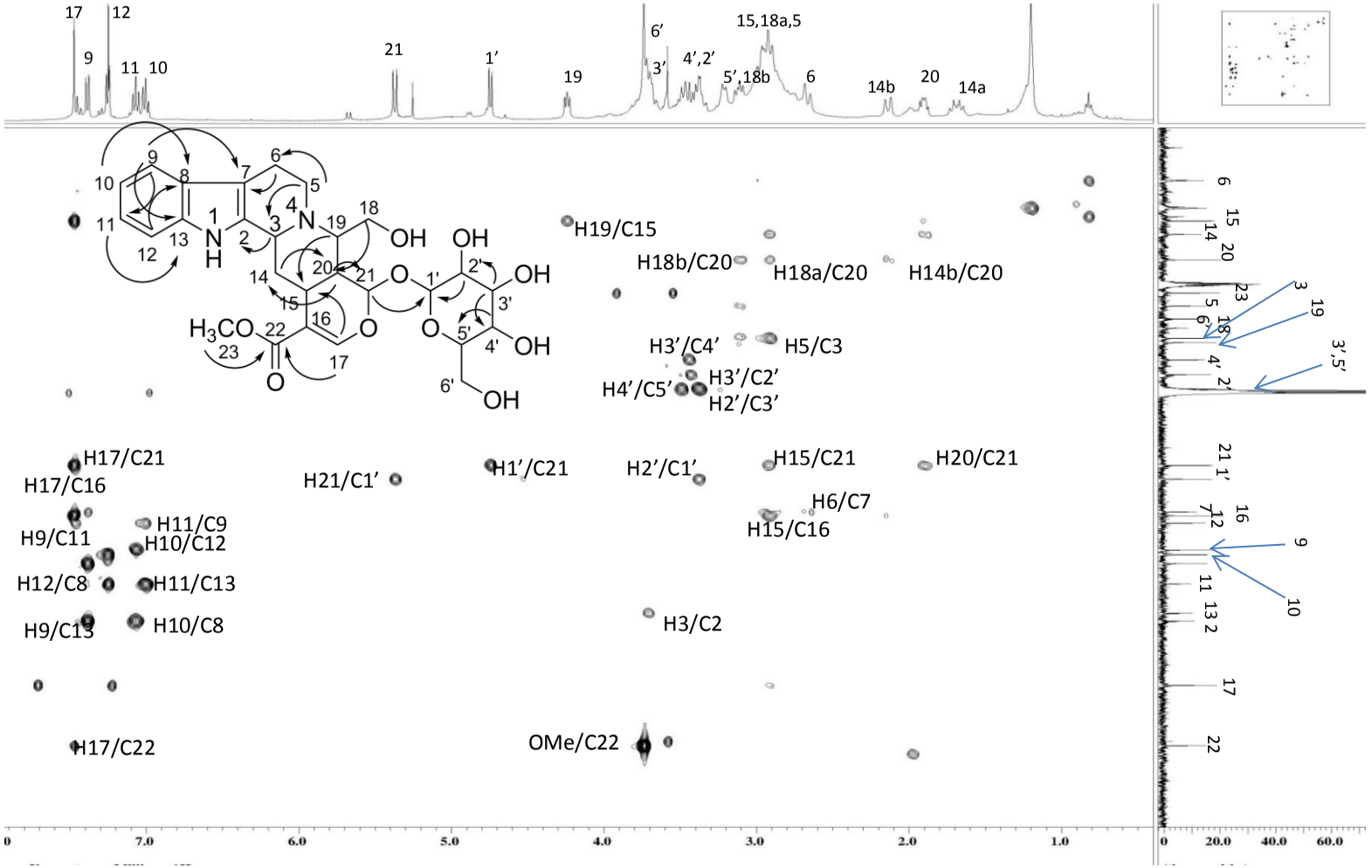


Figure 3.30: HMBC Spectrum of Compound **D**

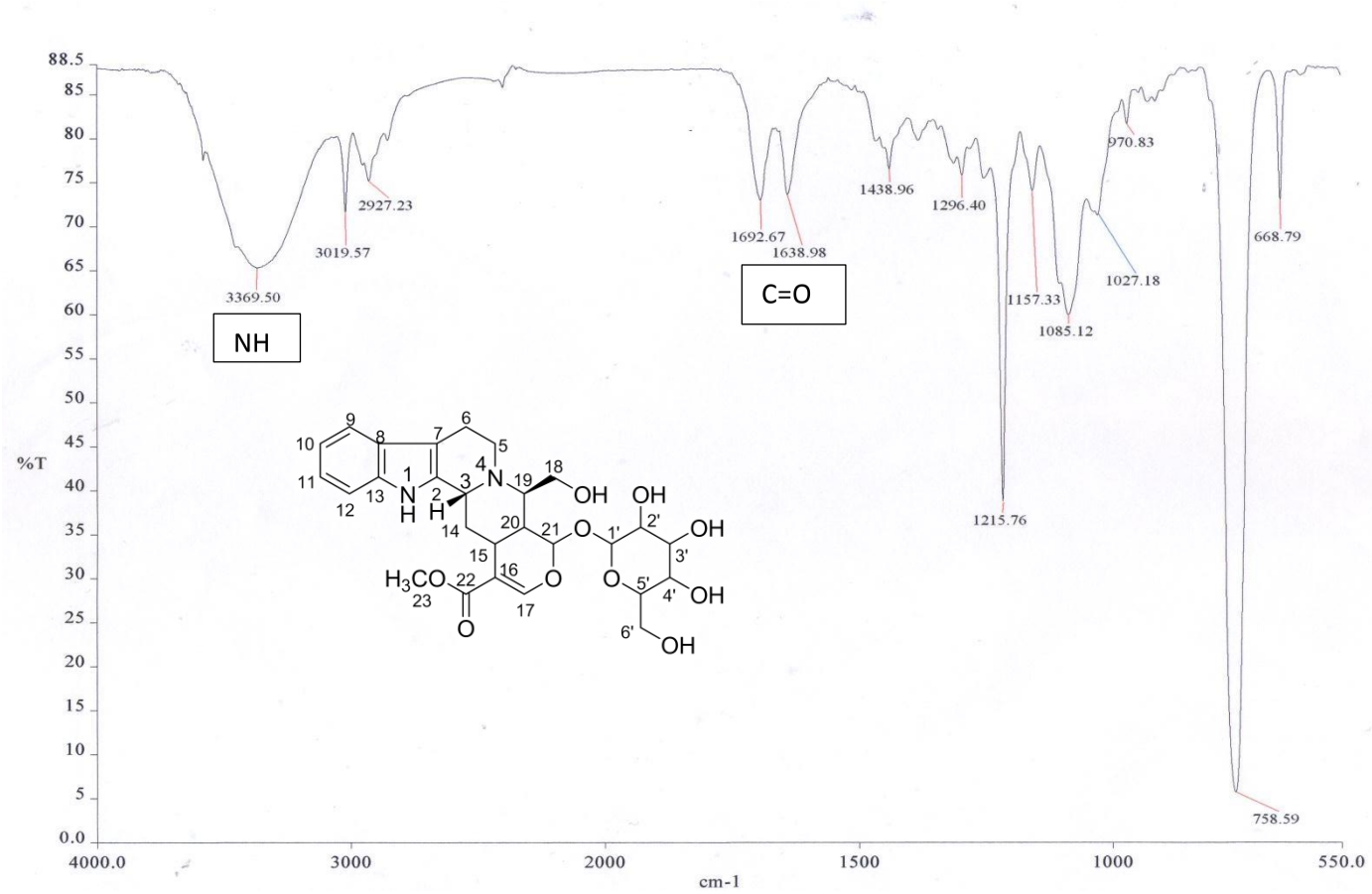
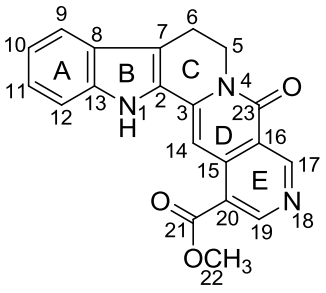


Figure 3.31: IR Spectrum of Compound **D**

3.1.5 Compound E: Neonaucline 79



79

Compound **E** was yielded as a yellowish amorphous solid. The UV spectrum exhibited maxima at 376, 284 and 204 nm which characteristic of an indole indole chromophore.[57-59] The IR spectrum (Figure 3.38) revealed absorption bands at 3364 and 1731 cm^{-1} for the stretching vibrations of NH and CO groups respectively. The LCMS-IT-TOFF spectrum (Figure 3.32) of compound **E** showed a pseudomolecular ion peak, $[M+H]^+$ at *m/z* 346.1140, that corresponded to the molecular formula of $C_{20}H_{15}N_3O_3$, suggested the presence of fifteen degrees of unsaturation with five ring and 10 double bonds.

In the ^{1}H-NMR spectrum (Figure 3.33), signals for seven aromatic protons due to one methoxy singlet and one $–CH_2$-CH_2-N- group were observed, thus suggesting an indolopyridinequinolizinone type of skeleton.[28] Among the seven aromatic proton signals, two resonated as doublet of doublets (*dd*) at δ 7.35 and 7.18 (H-10 and H11), two doublets at δ 7.64 and 7.46 (H-9 and H-12), and three singlets at δ 7.88, 9.32 and 9.69 assignable to H-14, H-17, and H-19, respectively. Further analysis of the ^{1}H-NMR and ^{13}C-NMR spectra showed that Compound **E** is very similar to naucletine[62] except that the former revealed the presence of a singlet representing a methoxyl group at δ 4.00(H-22) and δ 52.5 (C-22).

Furthermore, the COSY spectrum (Figure 3.35) revealed cross peaks between; H-5 (δ 4.54) /H-6 (δ 3.19); H-9 (δ 7.64) /H-10 (δ 7.18); H-10 (δ 7.18) /H-11 (δ 7.35); H -11 (δ 7.35) /H-12 (δ 7.46).

The ^{13}C NMR spectrum (Figure 3.34) revealed the existence of 20 carbon signals due to eight quaternary carbons, seven methines, two methylenes, one methoxy group and two overlapped carbonyl groups at δ 166.4 for position C-21 and C-23. The position of COOMe attached to C-20 (δ 120.4) in ring E was confirmed based on the HMBC correlations of H-14/C-20 (δ 120.4), H-19/C-20 (δ 120.4), H-22/C-21(δ 166.4), and H-17/C-23 (δ 166.4), respectively. Other significant HMBC correlation could be observed in Figure (3.37).

The complete ^{1}H NMR, ^{13}C NMR and HMBC spectral assignments were summarized in Table 3.5. Based on the spectroscopic findings, Compound **E** was proposed as a new compound namely namely neonaucline **79**.[60]

Table 3.5: ^{1}H NMR, ^{13}C NMR and HMBC spectral data of Compound **E** in $CDCl_3$

Position	^{1}H (δ_H,$CDCl_3$,Hz)	^{13}C (δ_C,$CDCl_3$)	HMBC
N-H	8.72 *s*	-	
2	-	127.4	
3	-	138.2	
5	4.54 *t* *J*=6.8	40.7	3, 6, 7, 23
6	3.19 *t* *J*=6.8	19.4	2, 5, 7
7	-	116.9	
8	-	125.7	
9	7.64 *d* *J*=7.8	119.9	7, 11, 13
10	7.18 *dd* *J*=7.8, 7.8	120.9	8
11	7.35 *dd* *J*=7.8, 7.8	125.6	9, 13
12	7.46 *d* *J*=7.8	111.9	10, 11
13	-	138.6	
14	7.88 *s*	95.1	2, 3, 16
15	-	141.9	
16	-	117.8	
17	9.32 *s*	154.2	15, 19, 20, 23
19	9.69 *s*	155.4	15, 17, 20
20		120.4	
21	-	166.4	
22-OMe	4.00 *s*	52.5	21
23	-	166.4	

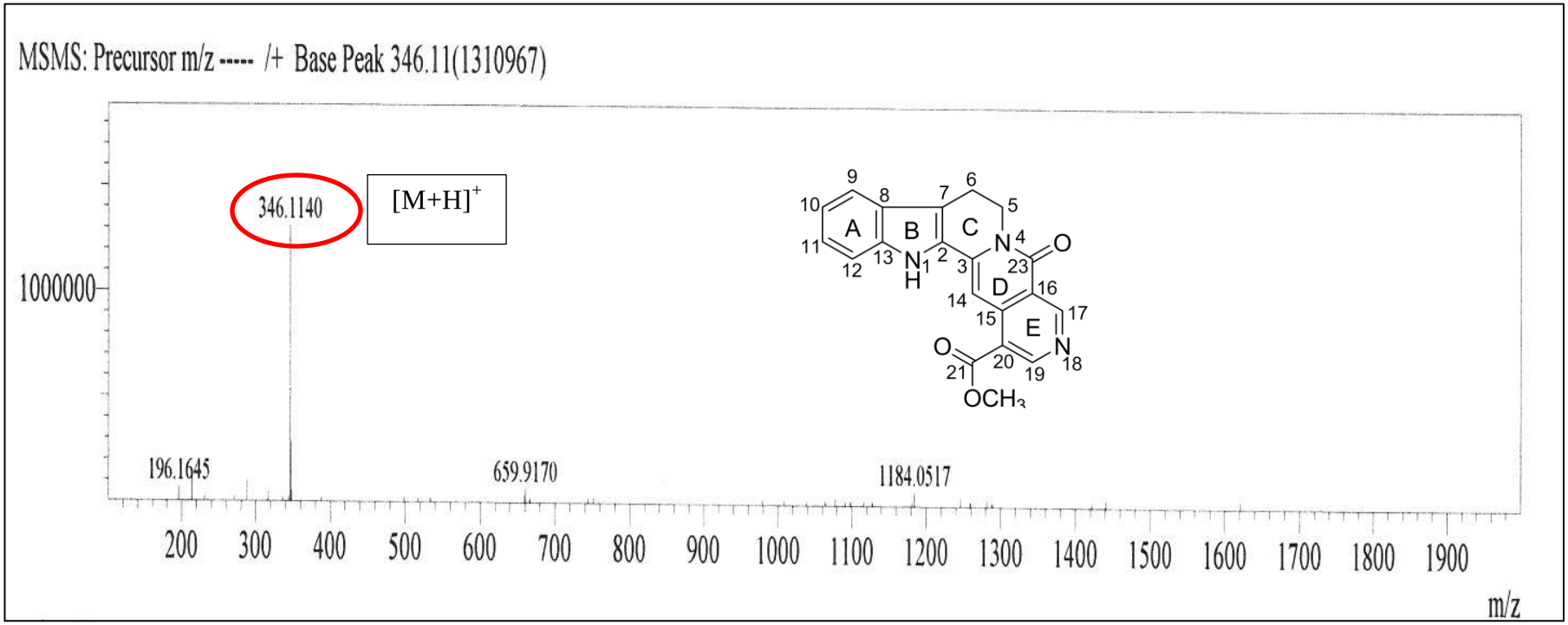


Figure 3.32: LCMS Spectrum of Compound **E**

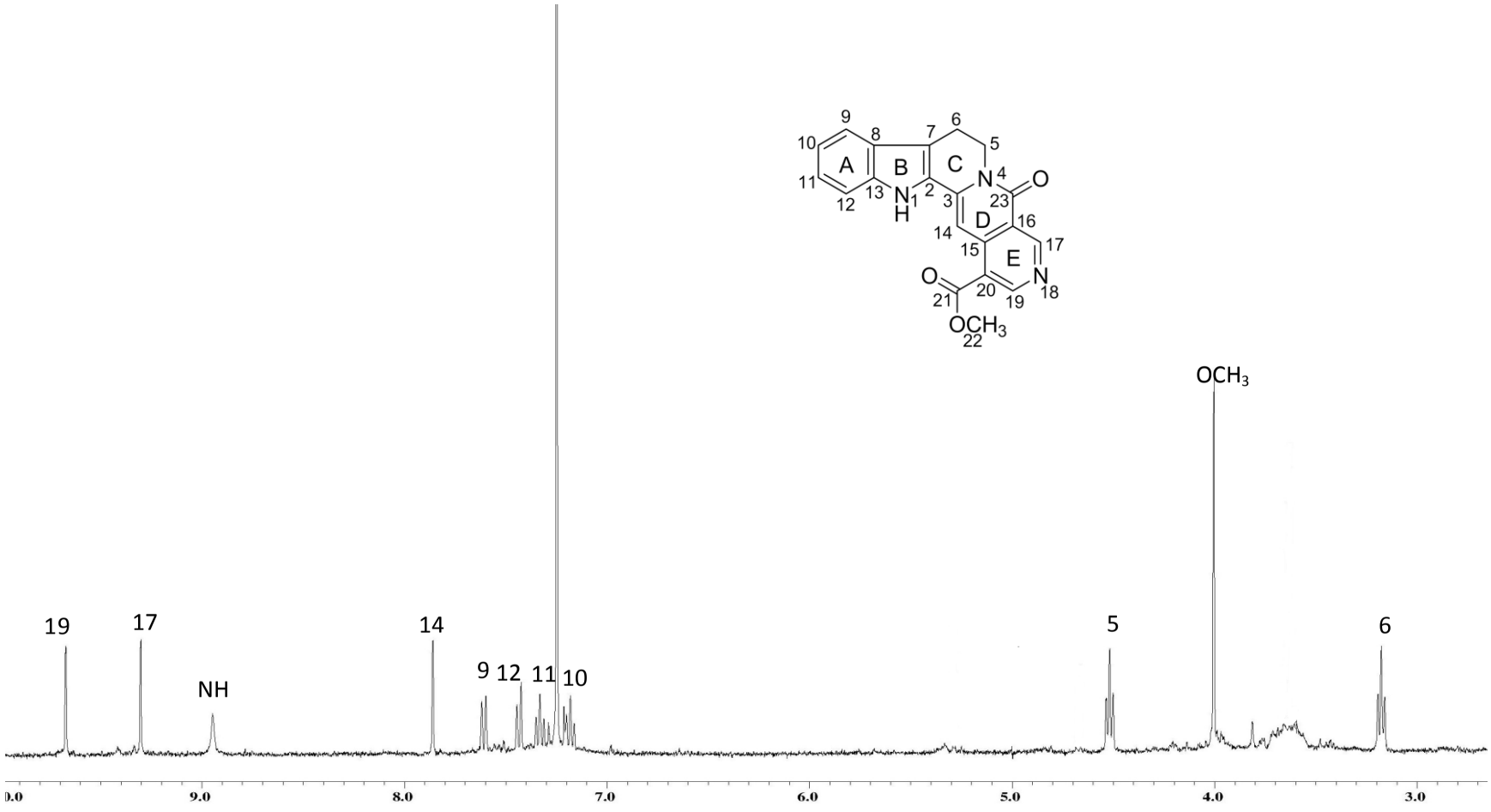


Figure 3.33: [1]HNMR Spectrum of Compound **E**

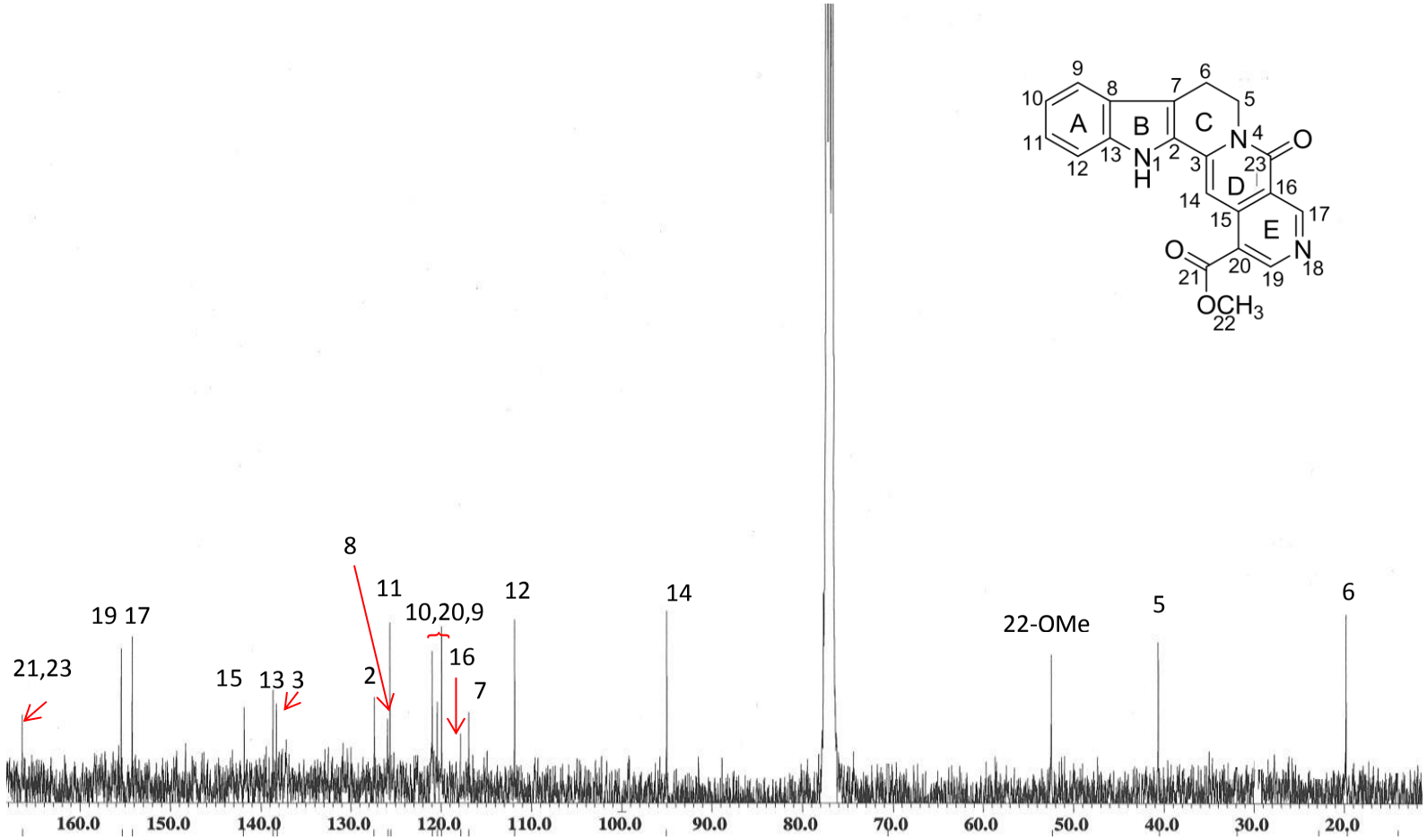


Figure 3.34: ^{13}C NMR Spectrum of Compound **E**

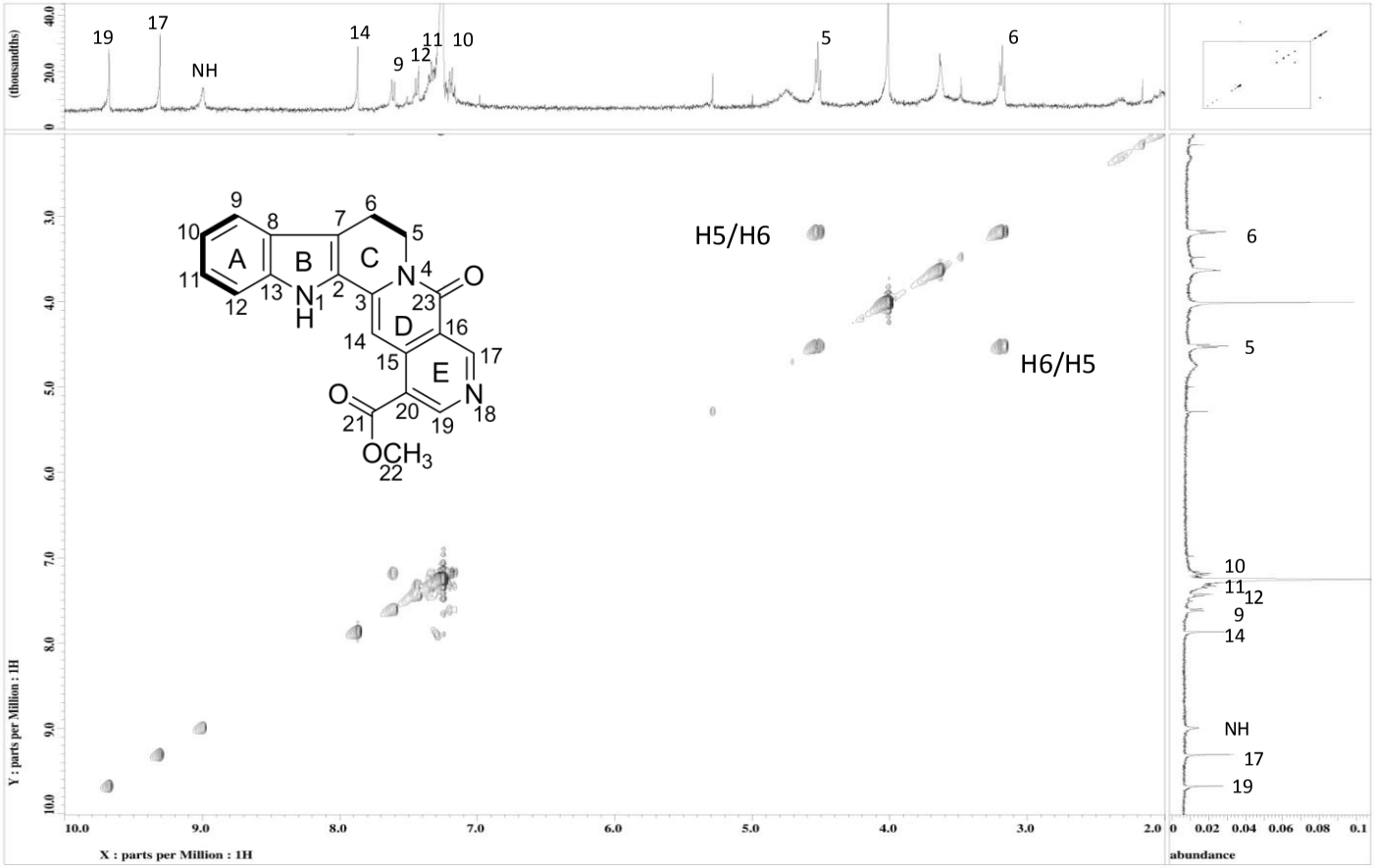


Figure 3.35: COSY Spectrum of Compound **E**

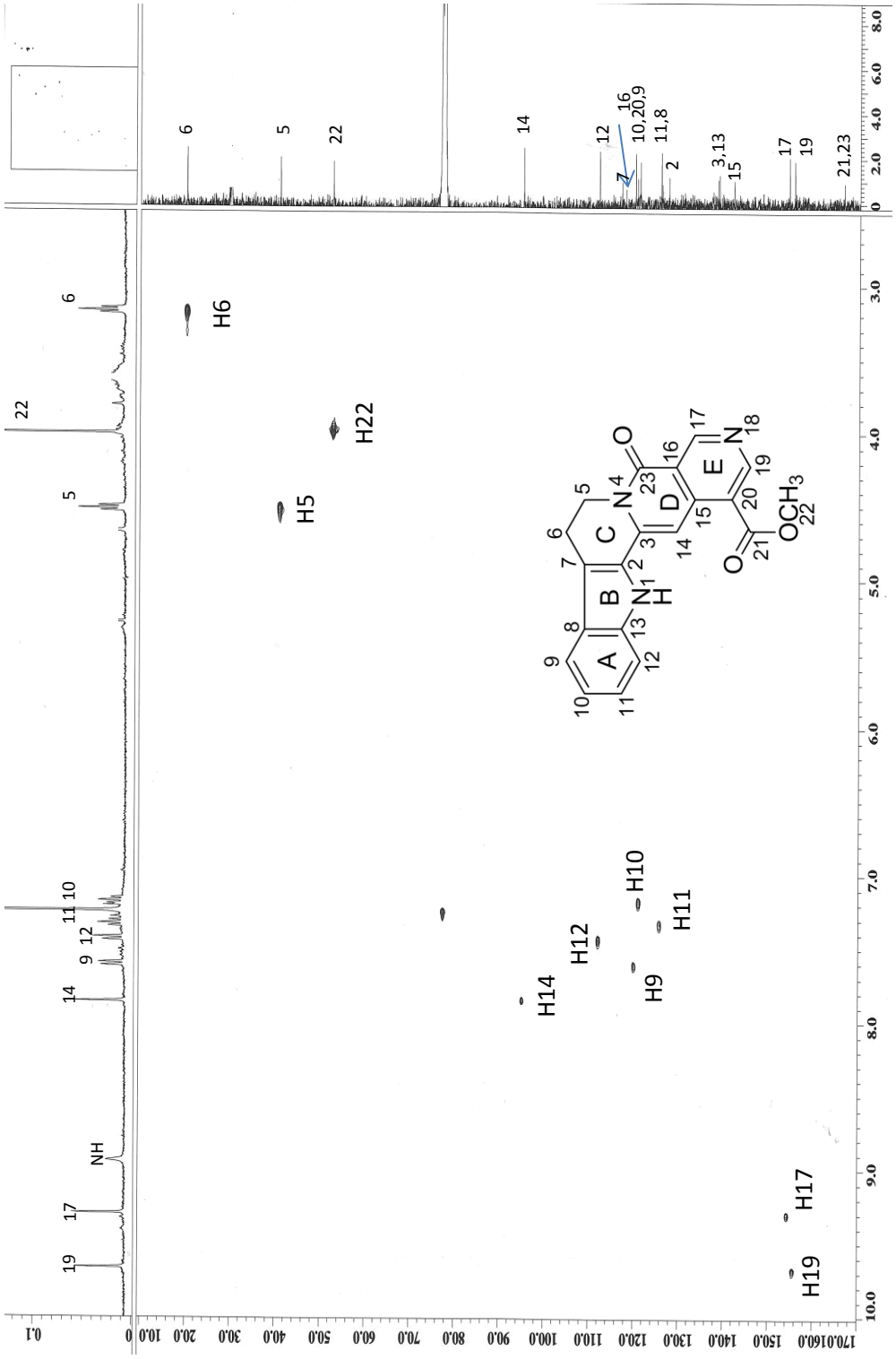


Figure 3.36: HMQC Spectrum of Compound **E**

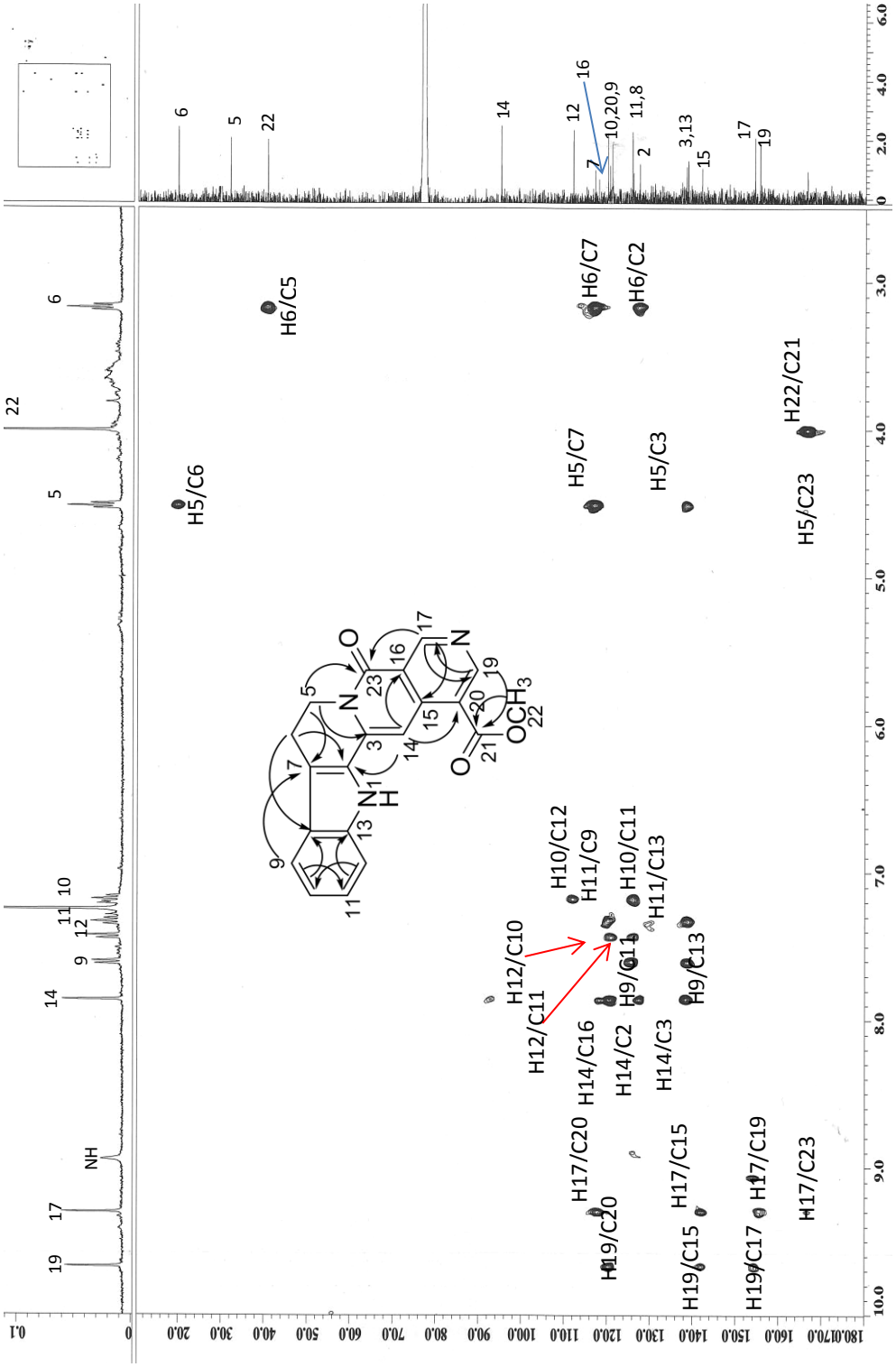


Figure 3.37: HMBC Spectrum of Compound **E**

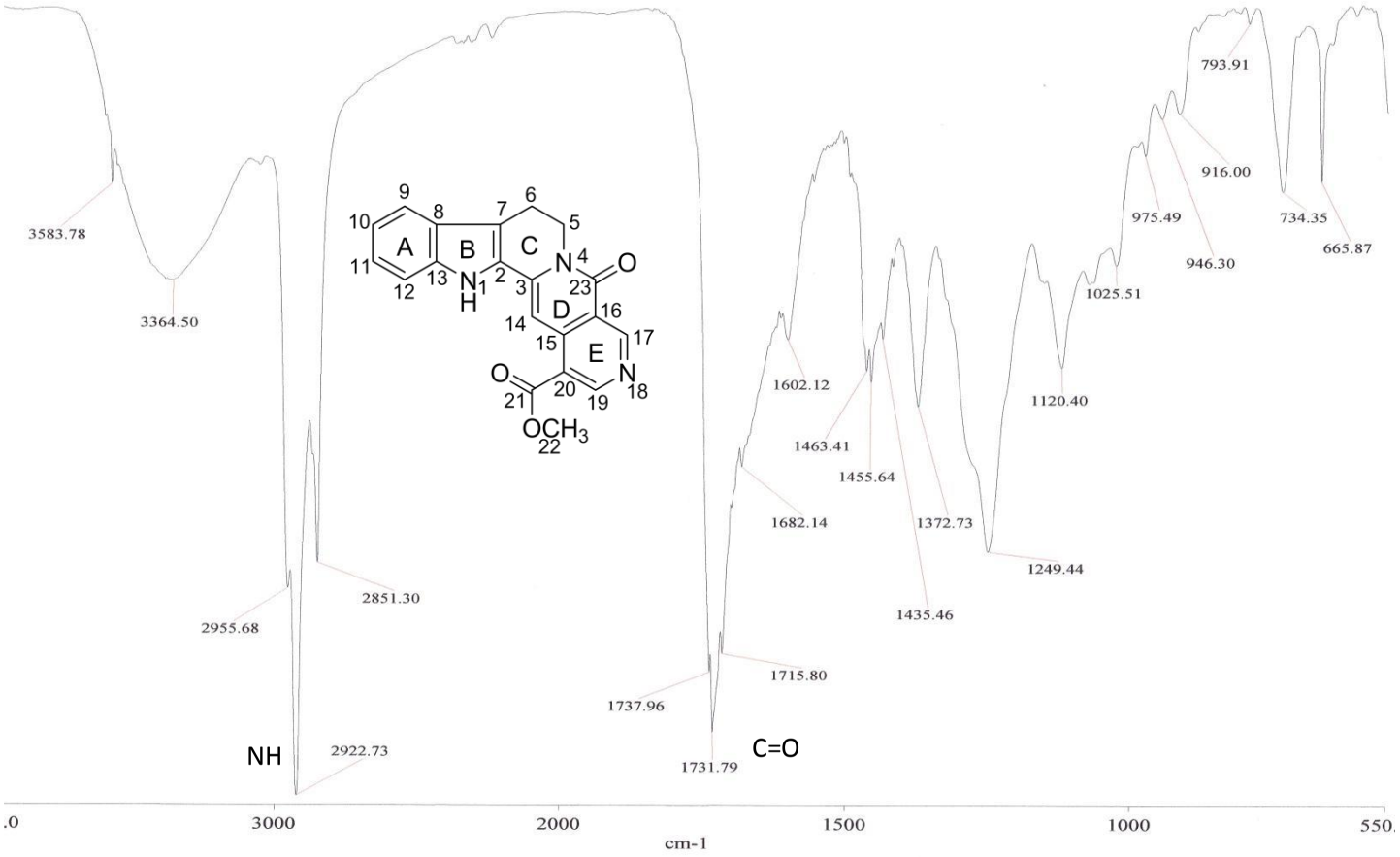
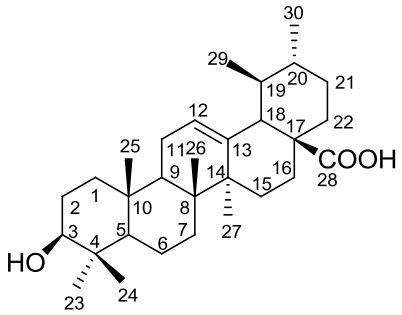


Figure 3.38: IR Spectrum of Compound **E**

3.1.6 Compound F: Ursolic Acid 73



73

Compound **F** was obtained as white amorphous powder. Its IR spectrum (Figured 3.45) showed the presence of hydroxyl functionality which appeared as a broad peak at 3421 cm^{-1}, while a strong sharped peak at 1639 cm^{-1} revealed the existence of a carbonyl group. The LCMS-IT-TOFF (Figure 3.39) showed a molecular ion peak at $[M+H]^+$ at *m/z* 457.2800, which corresponded to a molecular formula $C_{30}H_{48}O_3$ with seven degree of unsaturation. The UV spectrum displayed strong absorption at 472, 444, and 421 nm.

Furthermore, the ^{1}HNMR spectrum (Figured 3.40) presented at higher field, signals in the region δ 0.72 to 2.15, with seven methyl groups characteristic of a triterpene skeleton.[66] Compound **F** displayed five singlet signals corresponding to five tertiary methyl groups at δ 0.92 (H-23), 0.72 (H-24), 0.86 (H-25), 0.74 (H-26), 1.08 (H-27), and two secondary methyl groups reasonated as two doublets at δ 0.81 (H-29, *J*=6.0 Hz) and δ 0.89 (H-30, *J*=6.0 Hz) respectively. These signals were indicative of an ursane type.[52] In addition, an olefinic proton peak at δ 5.18 was assignable to H-12 which correlated to C-14 in HMBC spectrum, and one proton doublet at δ 2.15 (*J*=11.0Hz) was assigned to H-18 respectively, suggesting an urs-12-ene skeleton. A signal representing H-3 was observed at lower field at δ 3.14(*dd*, *J*=9.6, 6.4 Hz) due the attachment of a hydroxyl group to C-3.

The ^{13}CNMR spectrum (Figures 3.41) accounted for 30 carbon resonances, suggesting six quaternary, six methines, nine methylenes, seven methyls, one carboxylic and one carboxyl carbon, respectively. The peaks at δ 180.8 indicated the presence of a carbonyl group, assignable to C-28. A pair of sp^2 carbons (C-12 and C-13) showed their signals at δ 125.6 and 138.2 respectively. The prominent peaks at higher field; of δ 28.1, 15.6, 15.5, 23.6, 17.0, 16.9 and 21.2 were attributed to the methyl carbons of C-23, C-24, C-25, C-26, C-27, C-29 and C-30, respectively.

In addition, some of the important HMBC (Figure 3.43, 3.44) correlations were showed by the cross peak between methyl proton peaks at δ 0.92 (H-23) and 0.72 (H-24) with carbon δ 39.1 (C-4). Proton signals at δ 0.92 (H-29) and δ 0.92 (H-30) also correlated to carbon signals at δ 0.92 (C-19) and δ 0.92 (C-19) thus confirmed both methyl group were secondary which agreement with ursane type.

The complete assignment of NMR data is displayed in Table 3.6. On the basis of these spectral evidence and comparison with those literature literature values[51, 52, 56] compound **F** was characterized as the well-known ursolic acid **73**.

Table 3.6: ^{1}HNMR and ^{13}CNMR (δppm) spectral data of Compound **F** in $CDCl_3$

Position	^{1}H (δ_H, Hz $CDCl_3$)	^{13}C ($\delta_{C\ ppm,}$ $CDCl_3$)
1	0.89-0.86 *m*	38.6
2	1.08-1.02 *m*	28.1
3	3.14 *dd J*=1, 6.4	78.9
4	-	39.5
5	0.68 *d J*=10.0	55.3
6	1.49-1.45 *m*	18.3
7	1.39-1.26 *m*	33.1
8	-	39.1
9	1.49-1.44 *m*	47.8
10	-	36.8
11	1.85-1.81 *m*	23.3
12	5.18 *m*	125.6
13	-	138.2
14	-	42.1
15	1.64-1.57 *m*	26.9
16	1.60-1.53 *m*	24.2
17	-	47.6
18	2.15 *d J*=11.0	52.8
19	1.30-1.28 *m*	38.9
20	0.97-0.92 *m*	38.7
21	1.44-1.42 *m*	30.7
22	1.67-1.60 *m*	36.9
23	0.92 *s*	28.1
24	0.72 *s*	15.6
25	0.86 *s*	15.5
26	0.74 *s*	17.0
27	1.08 *s*	23.6
28	-	180.8
29	0.81 *d J*= 6.0	16.9
30	0.89 *d J*= 6.0	21.2

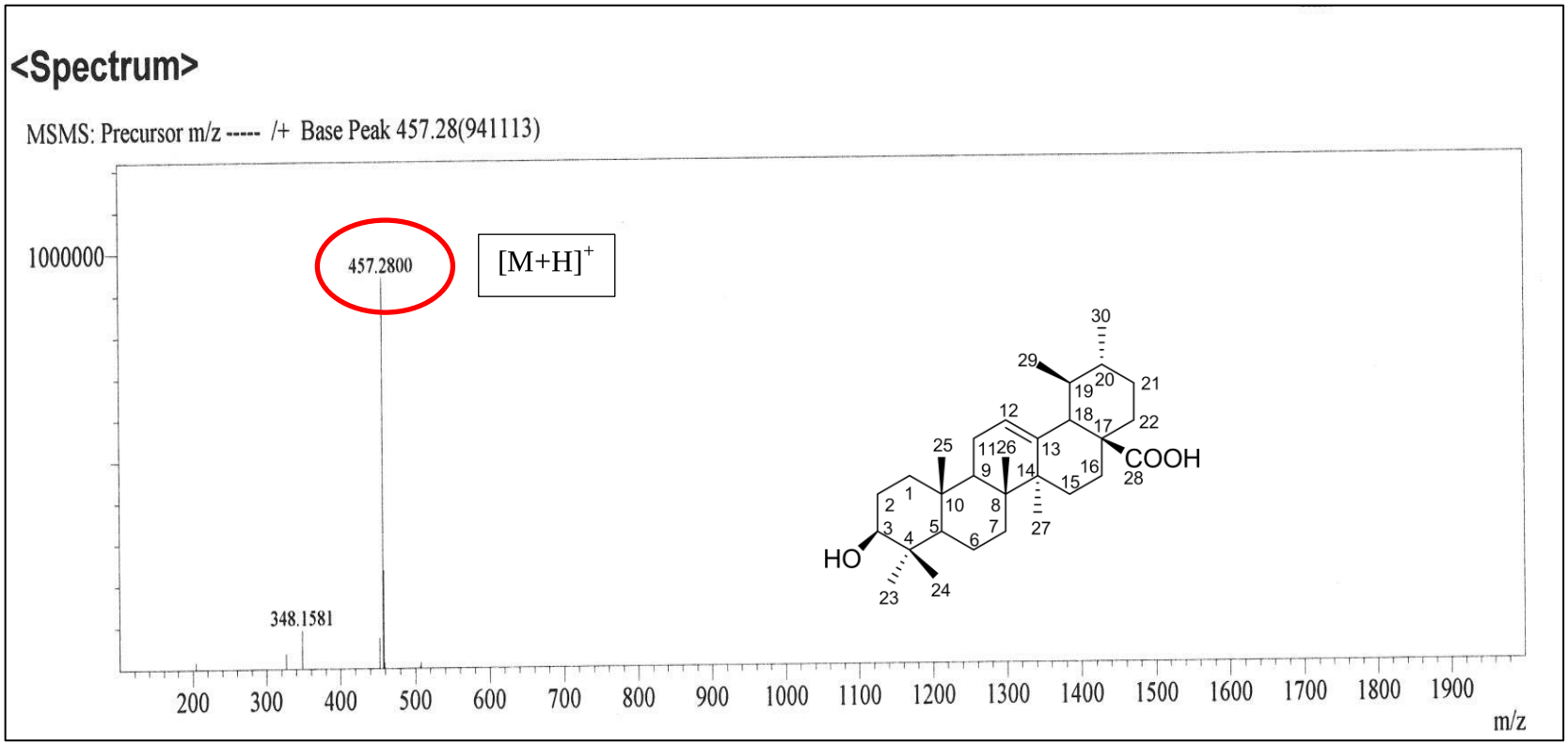


Figure 3.39: LCMS Spectrum of Compound **F**

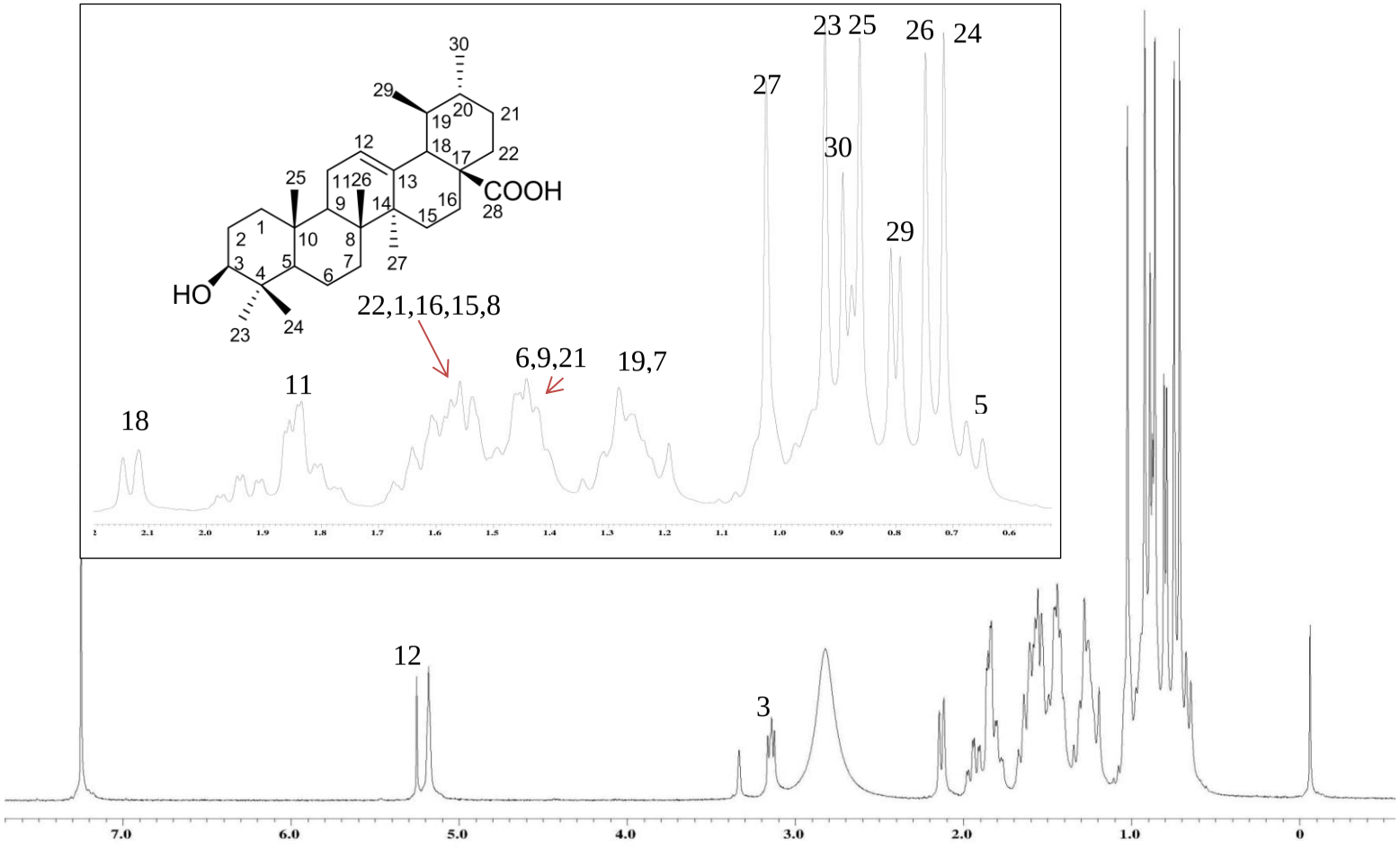


Figure 3.40: [1]HNMR Spectrum of Compound **F**

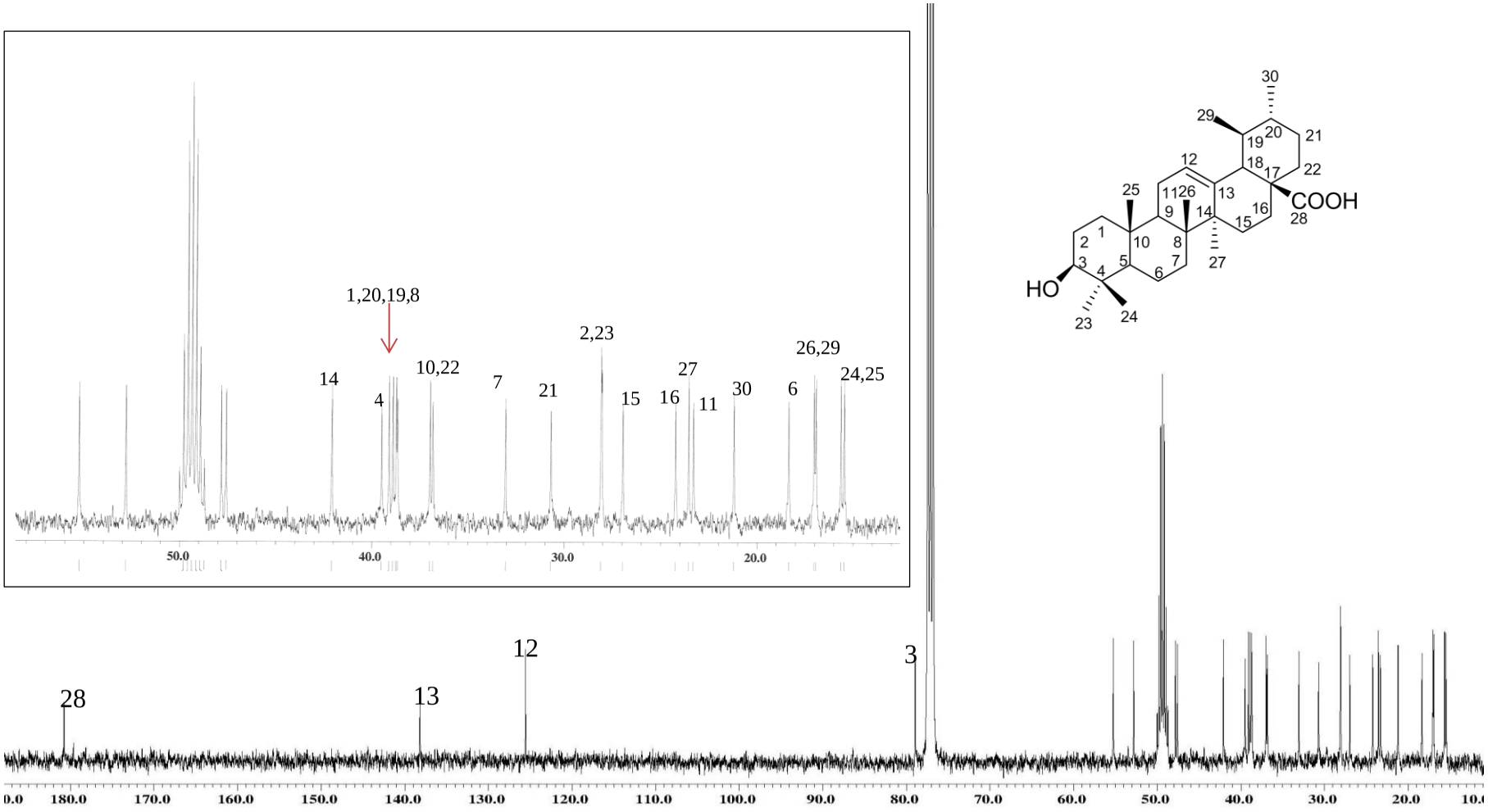


Figure 3.41: ^{13}CNMR Spectrum of Compound **F**

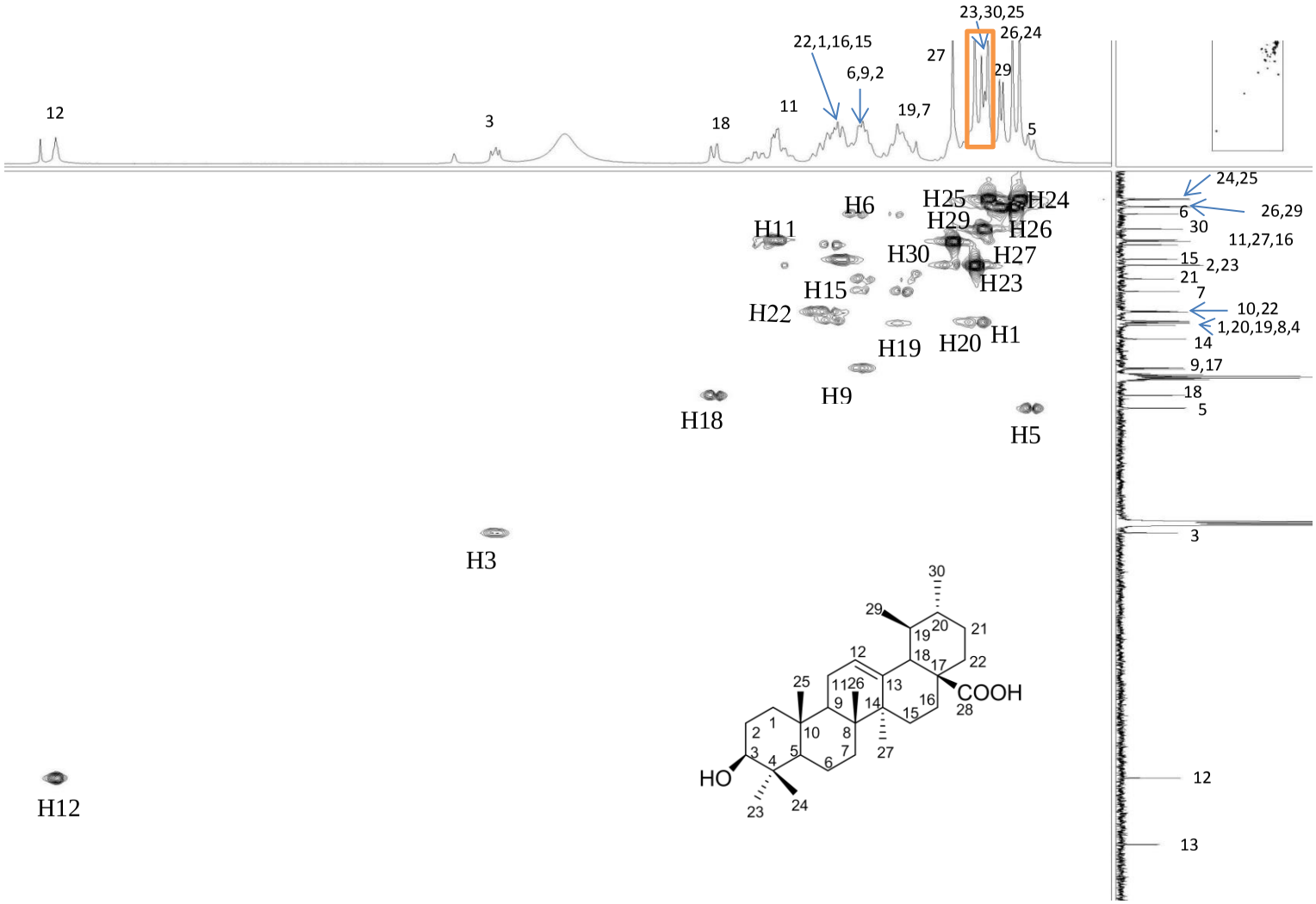


Figure 3.42: HMQC Spectrum of Compound **F**

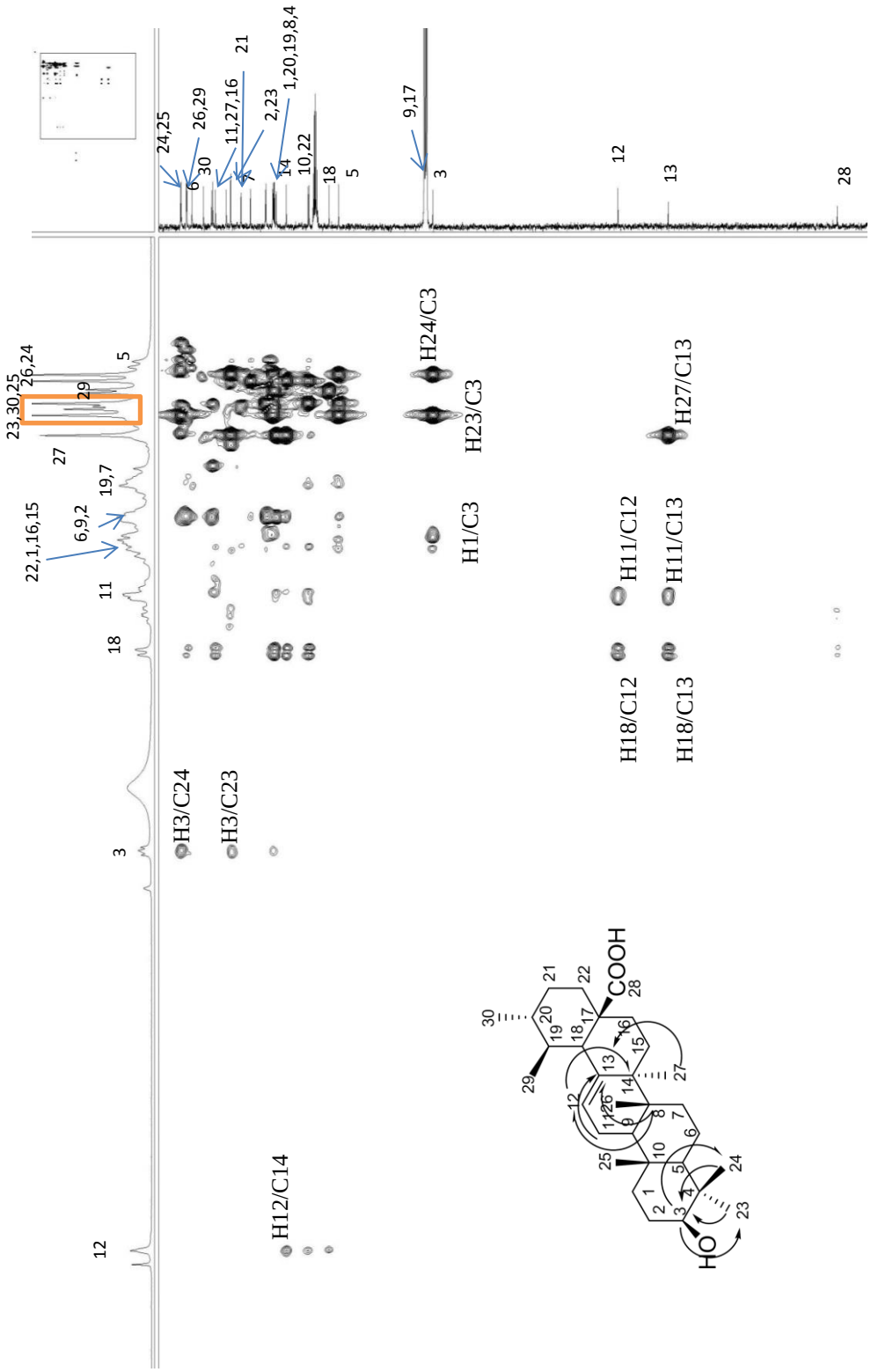


Figure 3.43: HMBC Spectrum of Compound **F**

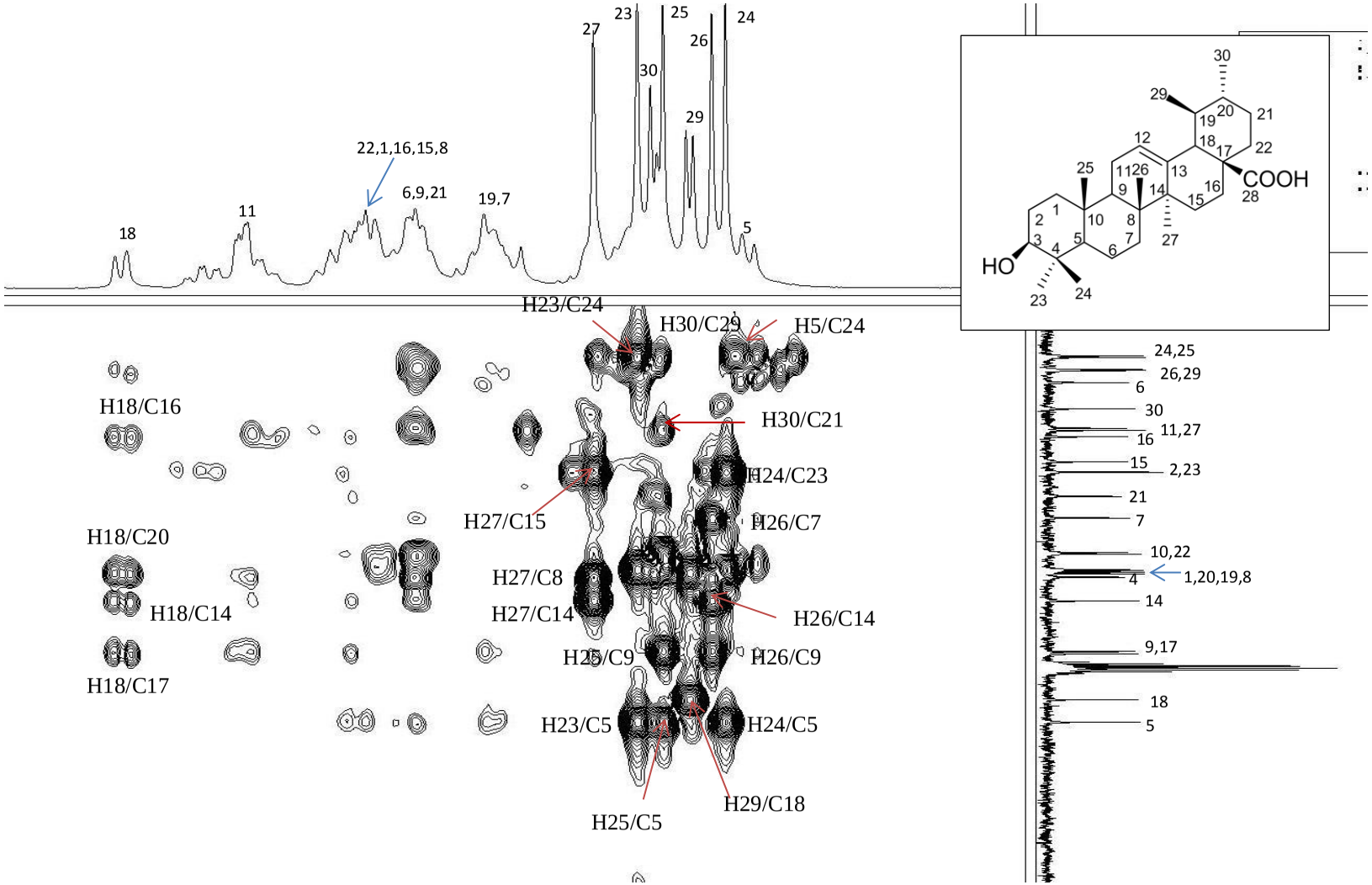


Figure 3.44: HMBC spectra of compound **F** (expansion)

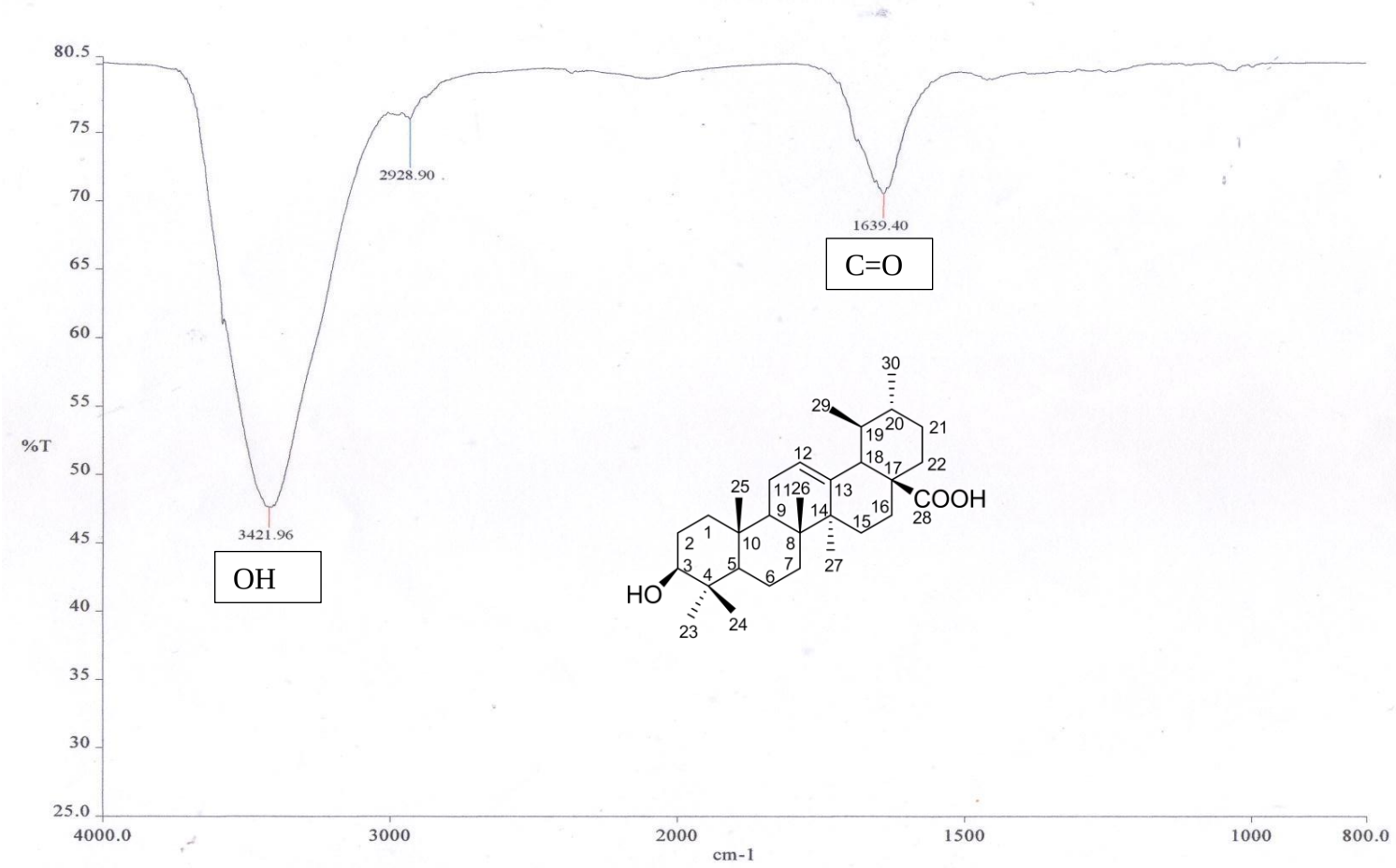
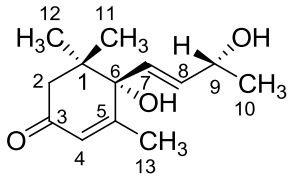


Figure 3.45: IR Spectrum of Compound **F**

3.1.7 Compound G: Blumenol A 75



75

Compound **F** $[\alpha]^{25}_{D}$ + 99.8° (c 0.65, $CHCl_3$) was afforded as a yellowish amorphous powder. Strong absorption at 237 nm showed in the UV (MeOH) spectrum. The IR spectra (Figure 3.52) gave an absorption peak at 3413 and 1372 cm^{-1} indicating the presence of hydroxyl group and carbonyl group. The LCMS-IT-TOFF spectrum of (Figure 3.46) showed a pseudomolecular ion peak at $[M+H]^+$, *m/z* 247.1334 corresponding to molecular formula $C_{13}H_{20}O_3$.

^{1}H-NMR spectrum (Figure 3.47) showed the doublet of doublet peaks of a trans double bond at δ 5.79 and 5.85 with coupling constant *J*=15.7 Hz attributable to H-8 and H-7 respectively. A secondary methyl groups resonated as a broad doublet at δ 1.30 (*J*=6.30) was coupled to H-9 which appeared as a multiplet at δ 4.41, assigned to H-10 and H-9 respectively. Another three methyl groups exhibited broad singlets at δ 1.02, 1.08 and 1.90 representing CH_3-11, CH_3-12 and CH_3-13, in which CH_3-13 is more downfield because of the electron withdrawing effect of the adjacent OH group. A pair of methylene protons of C-2 appeared as doublet at δ 2.24 (*J*=17.1) and 2.45(*J*=17.1). The COSY (Figure 3.49) experiment led to assignments of the aliphatic protons with the significant correlations between H-2α (2.45) with H-2β (2.24), H-2β (2.24) with H-2α (2.45), H-7 (5.79) with H-8 (5.85) and H-8 (5.85) with H-7 (5.79).

The ^{13}C NMR spectrum (Figure 3.48) revealed 13 carbon signals due to four quaternary carbons, four methines, two methylenes, four methyl group and one carbonyl group suggesting a 13C blumenol type nor-isoprenoid skeletons.[54] Three oxygenated

carbon atoms were observed at δ 68.0, 79.0 and 198.0 indicating the presence of hydroxyl group at C-9 and C-6, and carbonyl group at C-3.

In HMBC spectrum showed correlations between H-2 with C-1, C-6, C-4 and C-3, and also correlation between H-4 with C-6 and C-13, thus confirm the hydroxylated buitenyl chain system. Other important correlations were showed in Figure 3.51. The complete data of ^{1}H, ^{13}C NMR and HMBC were summarized at Table 3.7.

The analysis of the spectroscopic data obtained and comparison with literature values[67, 68, 69, 54], strongly suggested that compound **G** is blumenol A **75**. Blumenol A **75** was first isolated from the leaves *Podocarpus blumei.* [54]

Table 3.7: ^{1}H-NMR, ^{13}C-NMR and HMBC spectral data for Compound **G** in $CDCl_3$

Position	^{1}H (δ_H,$CDCl_3$,Hz)	^{13}C (δ_C,$CDCl_3$)	HMBC
1	-	41.1	
2	2.24 *d J*=17.1	49.7	1,6,4,3
	2.45 *d J*=17.1		11,12,1,3
3	-	198.0	
4	5.90 *s*	126.9	13,6
5	-	162.7	
6	-	79.0	
7	5.79 *d J*=15.7	135.8	
8	5.85 *d J*=15.7	129.0	
9	4.41*m*	68.0	7
10	1.30 *d J*=6.3	23.8	9,8
11	1.02 *s*	22.9	6,2,1,12
12	1.08 *s*	24.0	6,2,1,11
13	1.90 *s*	18.9	6,4,5,

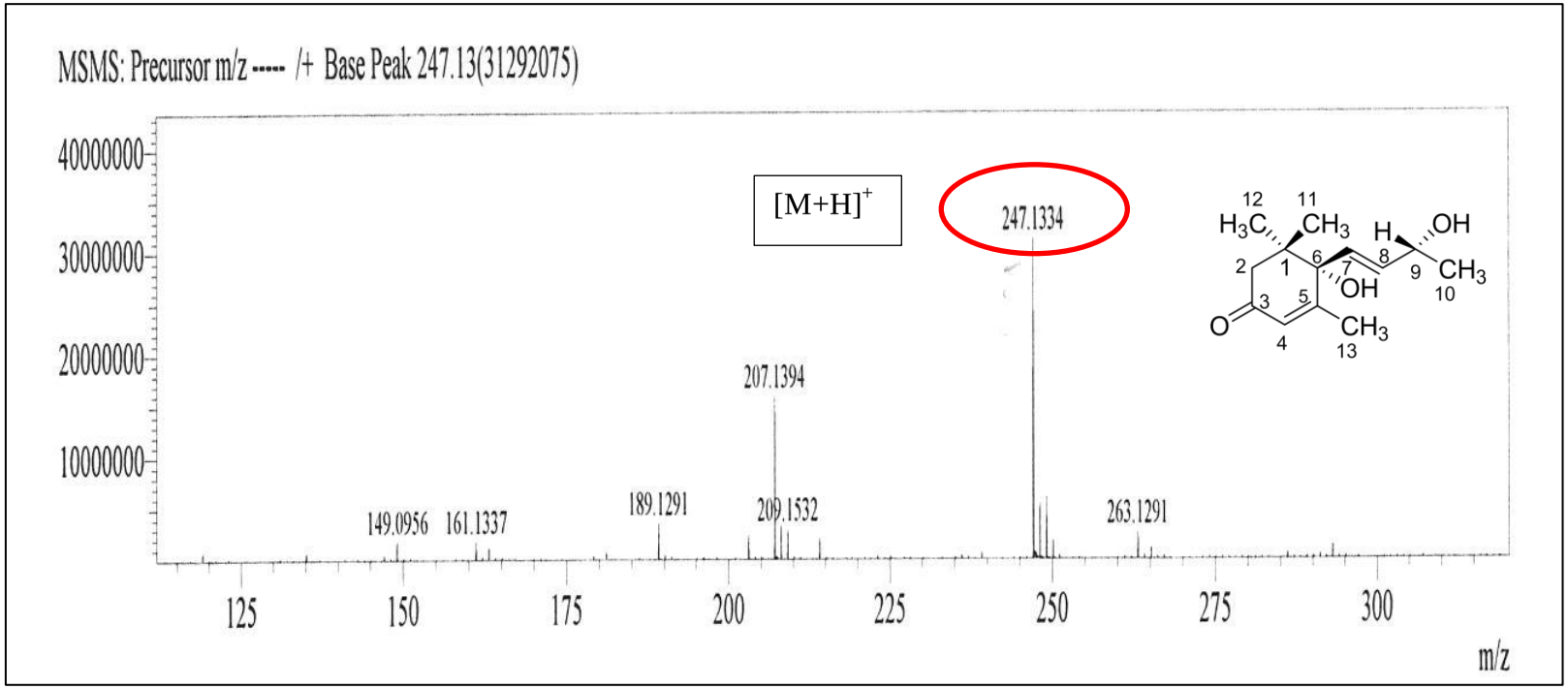


Figure 3.46: LCMS spectrum of Compound **G**

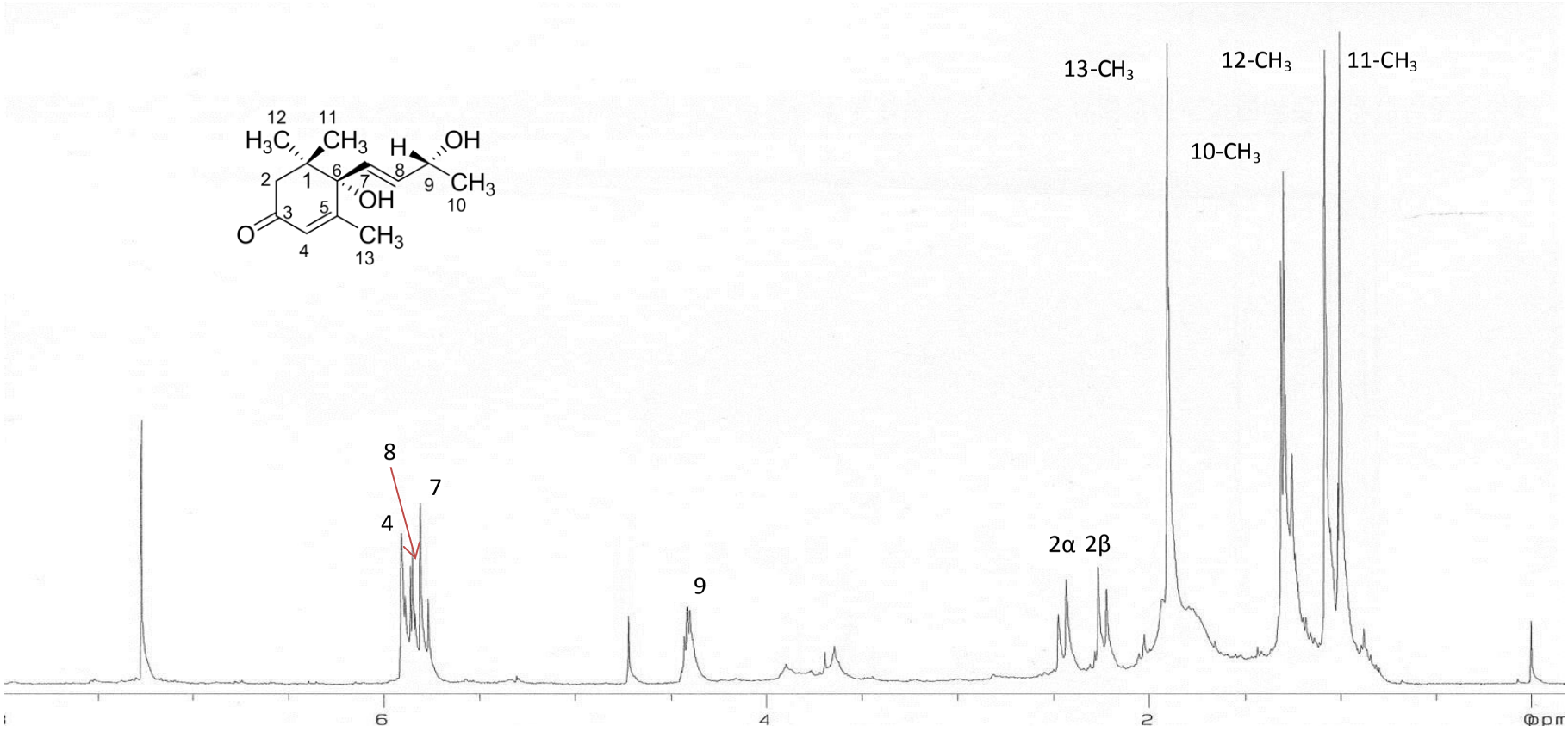


Figure 3.47: ^{1}H-NMR spectrum of Compound **G**

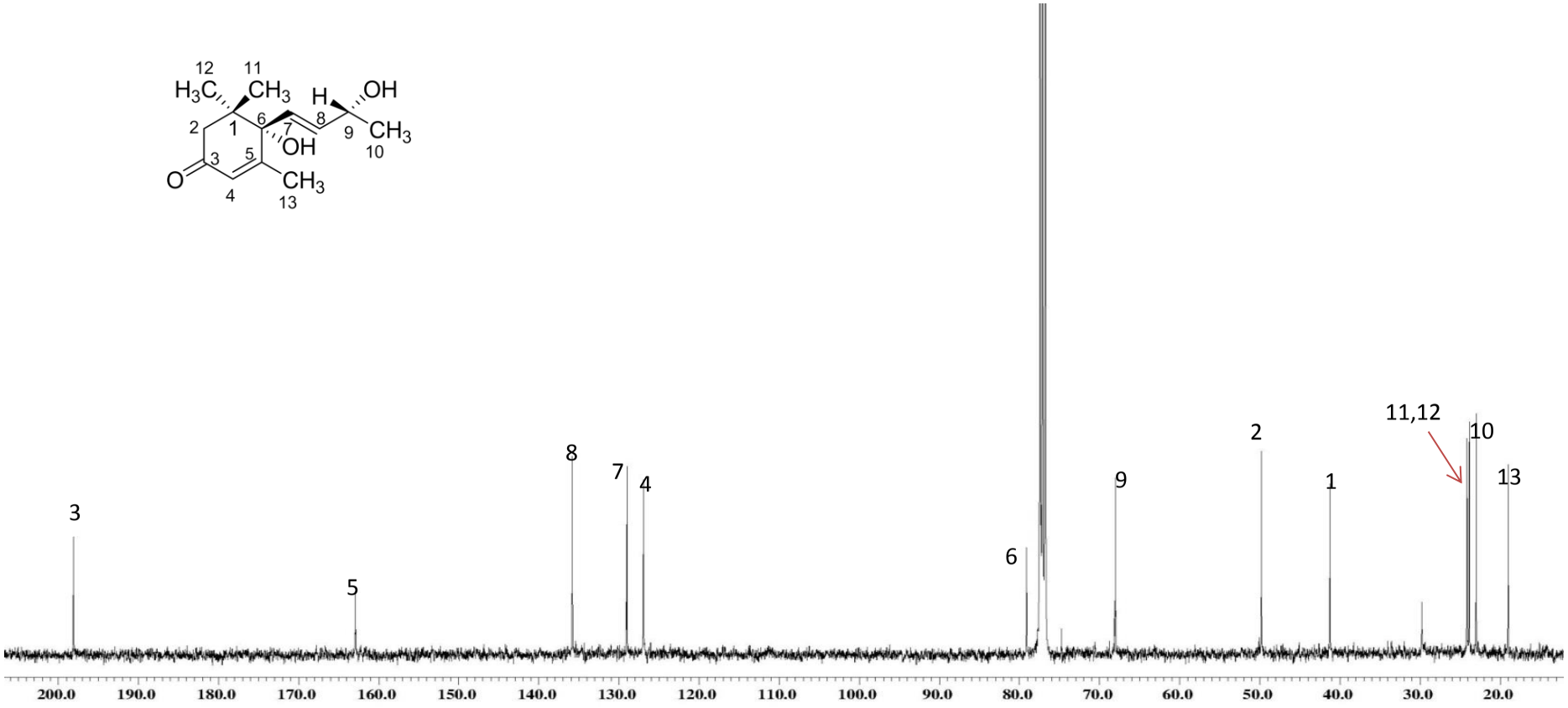


Figure 3.48: ^{13}C-NMR spectrum of Compound **G**

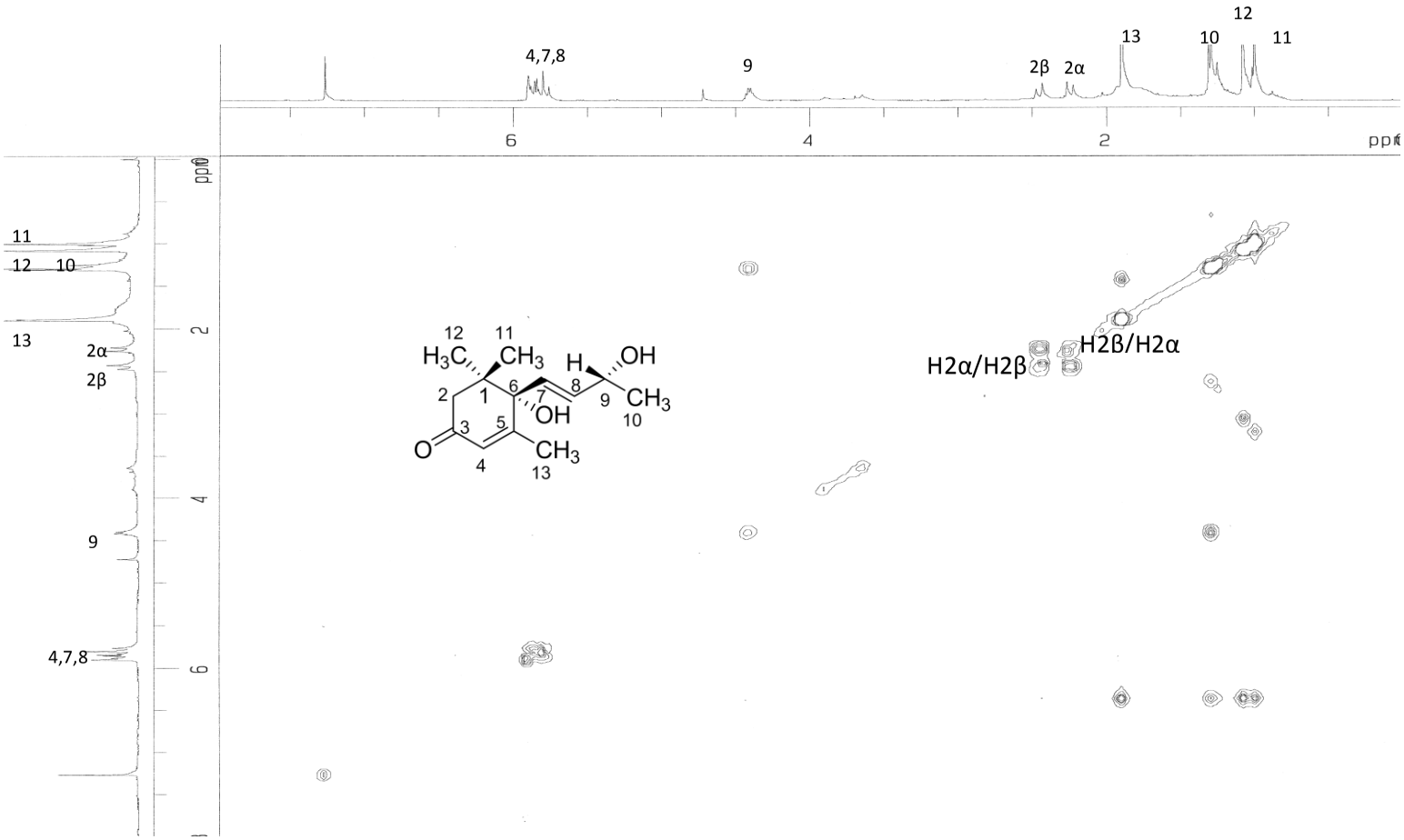


Figure 3.49: COSY spectrum of Compound **G**

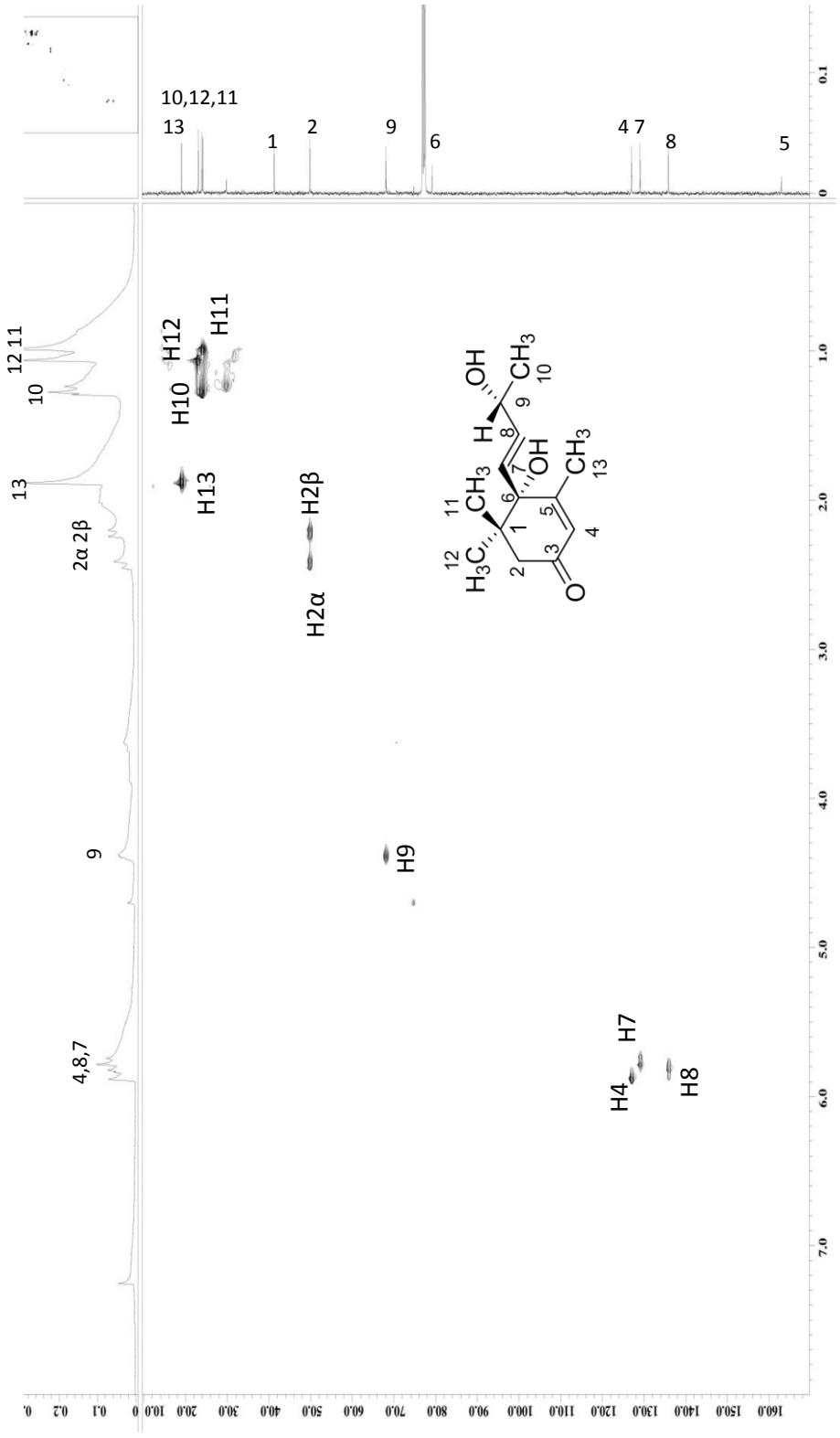


Figure 3.50: HMQC spectrum of Compound **G**

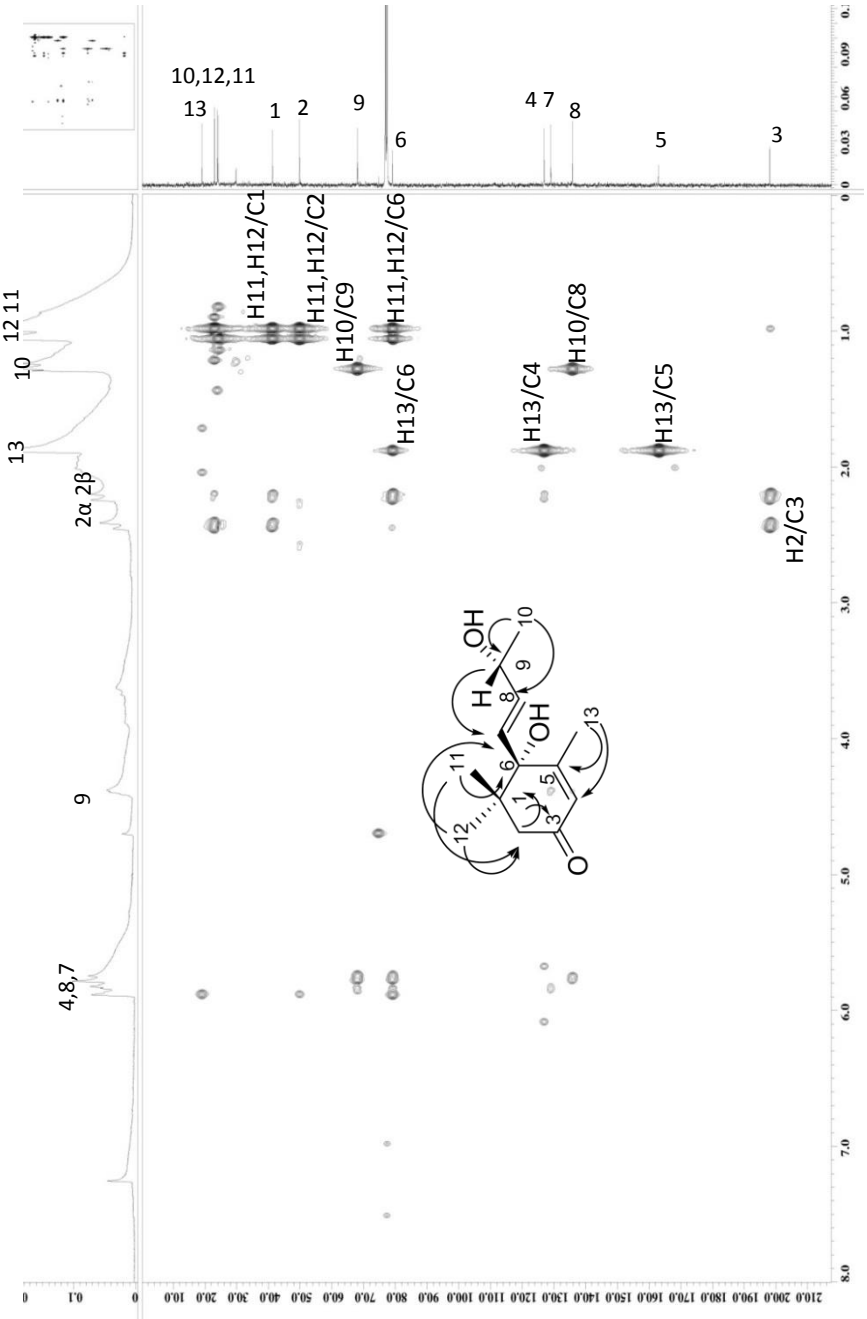


Figure 3.51: HMBC spectrum of compound **G**

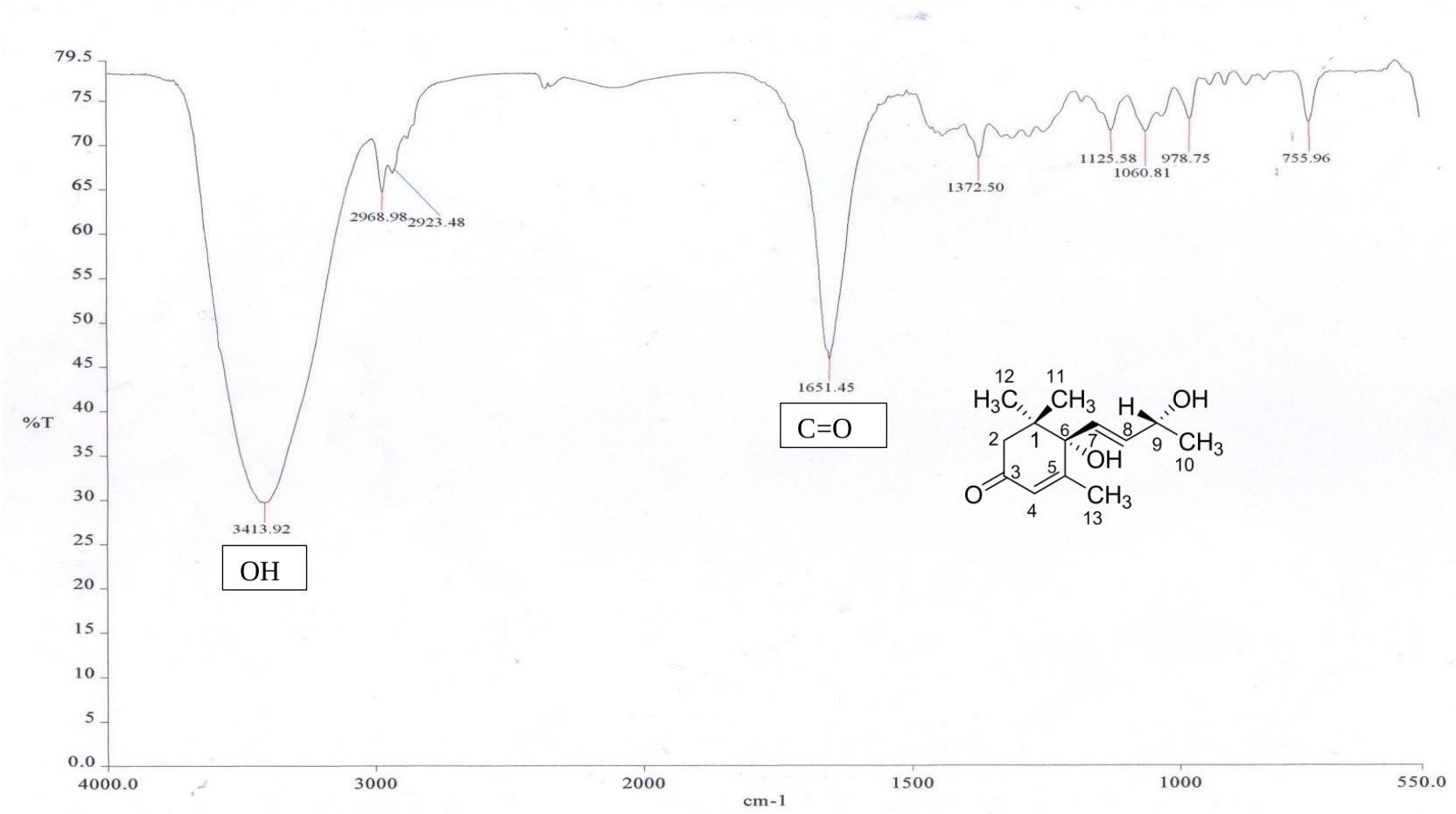
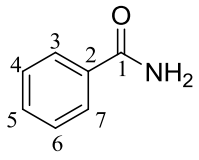


Figure 3.52: IR Spectrum of Compound **G**

3.1.8 Compound H: Benzamide 80



80

Compound **H** was isolted as crystalline needles with melting point 125-127°C. The UV spectrum showed maxima at 294 and 280 nm. The IR spectrum exhibited a strong peak at 1646 cm^{-1} attributed to a conjugated carbonyl group. The peak at 3365 cm^{-1} indicated the binding of NH_2 in the structure. The molecular ion peak was observed from GCMS spectrum (Figure 3.53) at *m/z* 121 $[M+H]^+$, gave the possible molecular formula C_7H_7O. The other important peaks were observed at *m/z* 105 $[M-16]^+$ and 77 $[M-44]^+$, due to the loss of NH_2 and $CONH_2$, respectively.

The ^{1}H-NMR spectrum (Figure 3.54) showed two sets of triplet and one doublet signal at the aromatic proton region, suggesting that the ring was mono substituted. H-3 and H-7 overlapping signal appeared as doublet peak at δ 7.78 (*d, J*=7.32), while overlapping proton signal H-4 and H-6 appeared as triplet peak at δ 7.42 (*t, J*=7.3). In addition, a triplet peak at δ 7.49 (*t, J*=7.3) belonged to H-5 was also observed. The NH_2 broad singlet signal was revealed at δ 6.10.

The ^{13}C NMR spectrum (Figure 3.55) further confirmed the presence of seven carbon atoms. Two set of methine carbon peaks overlapped, which were C-3 with C-7: and C-4 with C-6 at δ 127.3 and 128.6, respectively. Another methine carbon exhibited at δ 133.4, was attributted to C-5. Two quaternary carbons C-1 and C-2 peak appeared at δ 169.4 and 131.9. Comparison of the spectroscopic data with the data from literature values[70], confirmed that compound **H** is known and identified as benzamide **77**.

Table 3.8: ^{1}HNMR, ^{13}CNMR (δppm) spectral data of Compound **H** in $CDCl_3$

Position	^{1}H (δ_H ,Hz)	^{13}C ($\delta_{C\ ppm}$)
NH	6.10 *br s*	-
1	-	169.4
2	-	133.4
3	7.78 *d J*=7.3	127.3
4	7.42 *t J*=7.3	128.6
5	7.49 *t J*=7.3	131.9
6	7.42 *t J*=7.3	128.6
7	7.78 *d J*=7.3	127.3

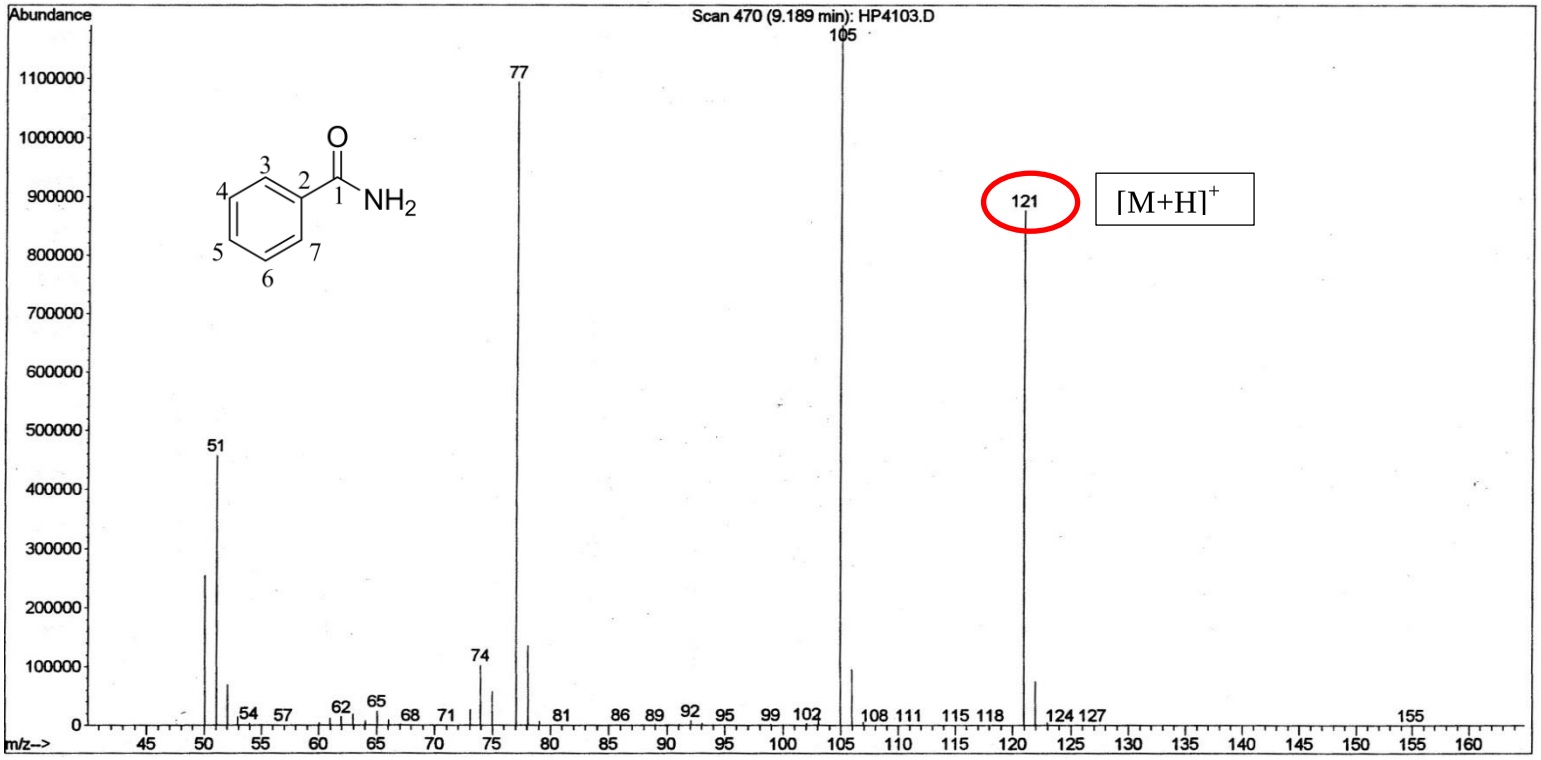


Figure 3.53: GCMS Spectrum of Compound **H**

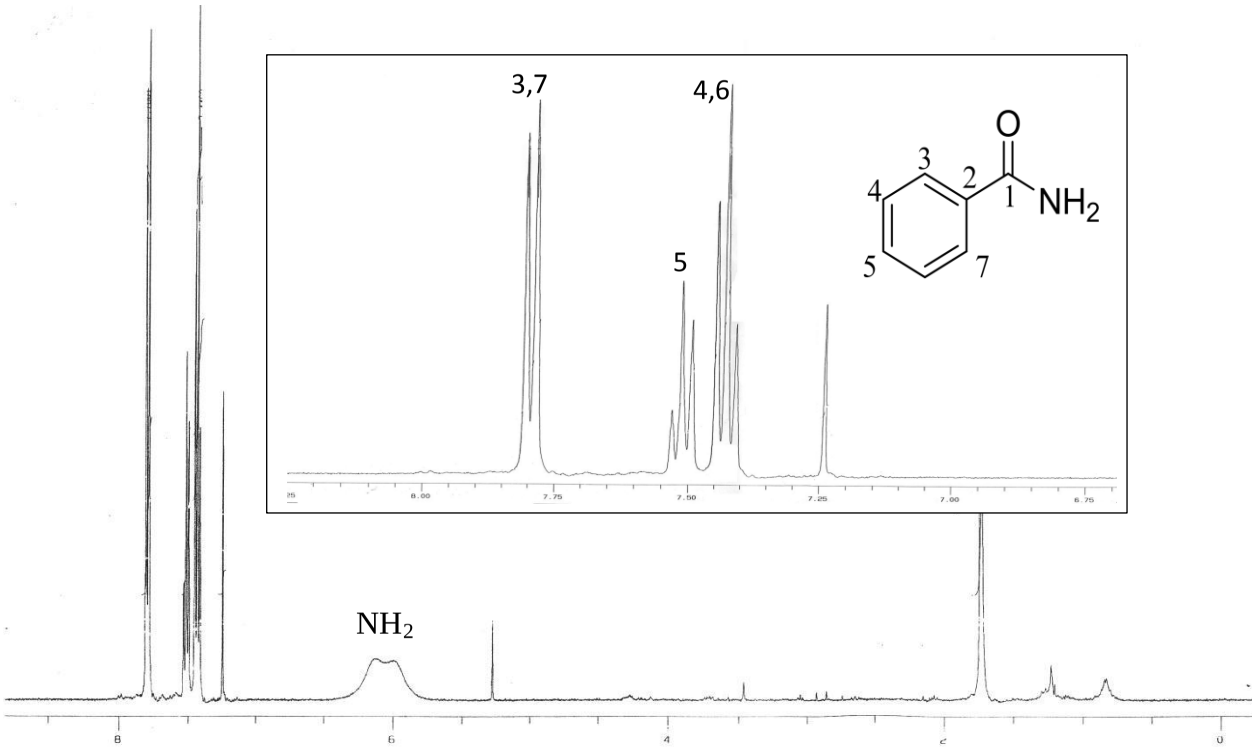


Figure 3.54: ^{1}HNMR Spectrum of Compound **H**

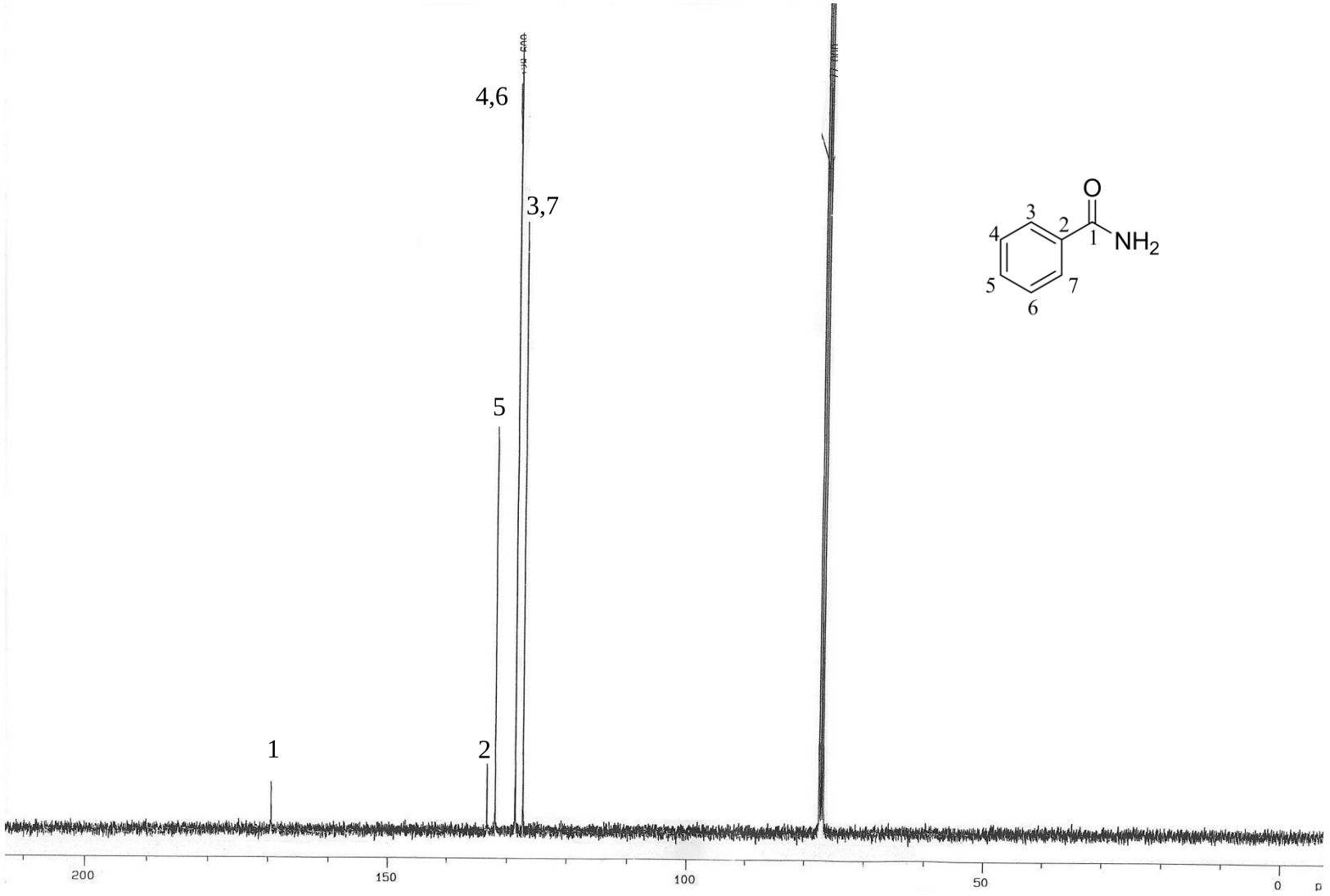
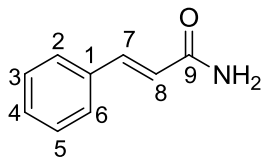


Figure 3.55: ^{13}CNMR Spectrum of Compound **H**

3.2.9 Compound I: Cinnamide 81



81

Compound **I** was afforded as crystalline needles with melting point 133-135 °C. The GCMS spectrum (Figure 3.56) for compound **I** was relatively simple with a molecular ion peak, $[M+H]^+$ observed at *m/z* 146 which correlated to the molecular formula C_9H_9NO. Other significant peak revealed at *m/z* 103 [M-44] and *m/z* 77 [M-69] indicating the loss of $CONH_2$ and $H_2C_2CONH_2$. The UV spectrum displayed the absorption bands at 217 and 272 nm. The IR spectrum (Figure 3.59) of this compound showed absorption peaks at 3368 and 1655 cm^{-1} indicated the presence of NH_2 group and CO stretching vibrations.

The ^{1}H NMR spectrum (Figure 3.57) signals at δ 6.46 (1H, *br d, J*= 16.04 Hz, H-8), δ 7.63 (1H, *br d, J*= 16.0 Hz, H-7), indicated the presence of a *trans*-substituted double bond. Signals at δ 7.36 (2H, *m*, H-2 & H-6), δ 7.50 (3H, *m*, H-3 , H4, H-5) in the ^{1}H NMR spectrum suggested the presence of mono substituted aromatic ring. The broad signals of NH_2 appeared at δ 5.95-6.10.

The ^{13}C NMR spectrum (Figure 3.58) is in agreement with the molecular formula deduced from the mass spectrum, accounting for all 9 carbons. There were two overlapping carbon peaks appearing at the same chemical shift at 128.0 (C-2 with C-6) and 128.9 (C-3 with C-5) which has equivalent environment in the aromatic ring. The complete NMR data of compound **I** was showed in Table 3.9. Comparison of all obtained data with the literature values[70] confirmed that compound **I** is known as cinnamide **81.**

Table 3.9: ^{1}HNMR, ^{13}CNMR (δppm) spectral data of Compound **I** in $CDCl_3$

Position	^{1}H (δ_H ,Hz)	^{13}C ($\delta_{C\ ppm}$)
1		134.6
2	7.36 *m*	128.0
3	7.50 *m*	128.9
4	7.50 *m*	130.1
5	7.50 *m*	128.9
6	7.36 *m*	128.0
7	7.46 *d J*=16.0	142.6
8	7.63 *d J*=16.0	119.6
9		168.1
NH_2	5.95 *s*	

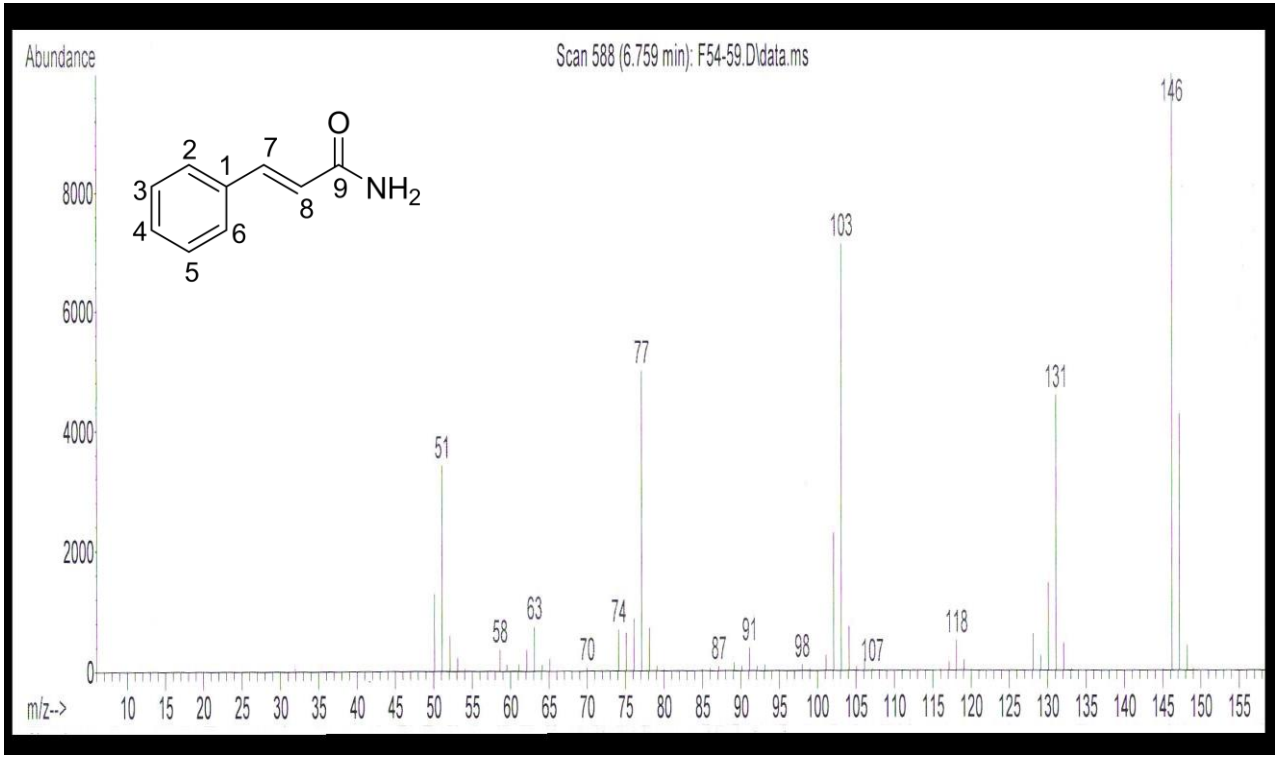


Figure 3.56: GCMS Spectrum of Compound **I**

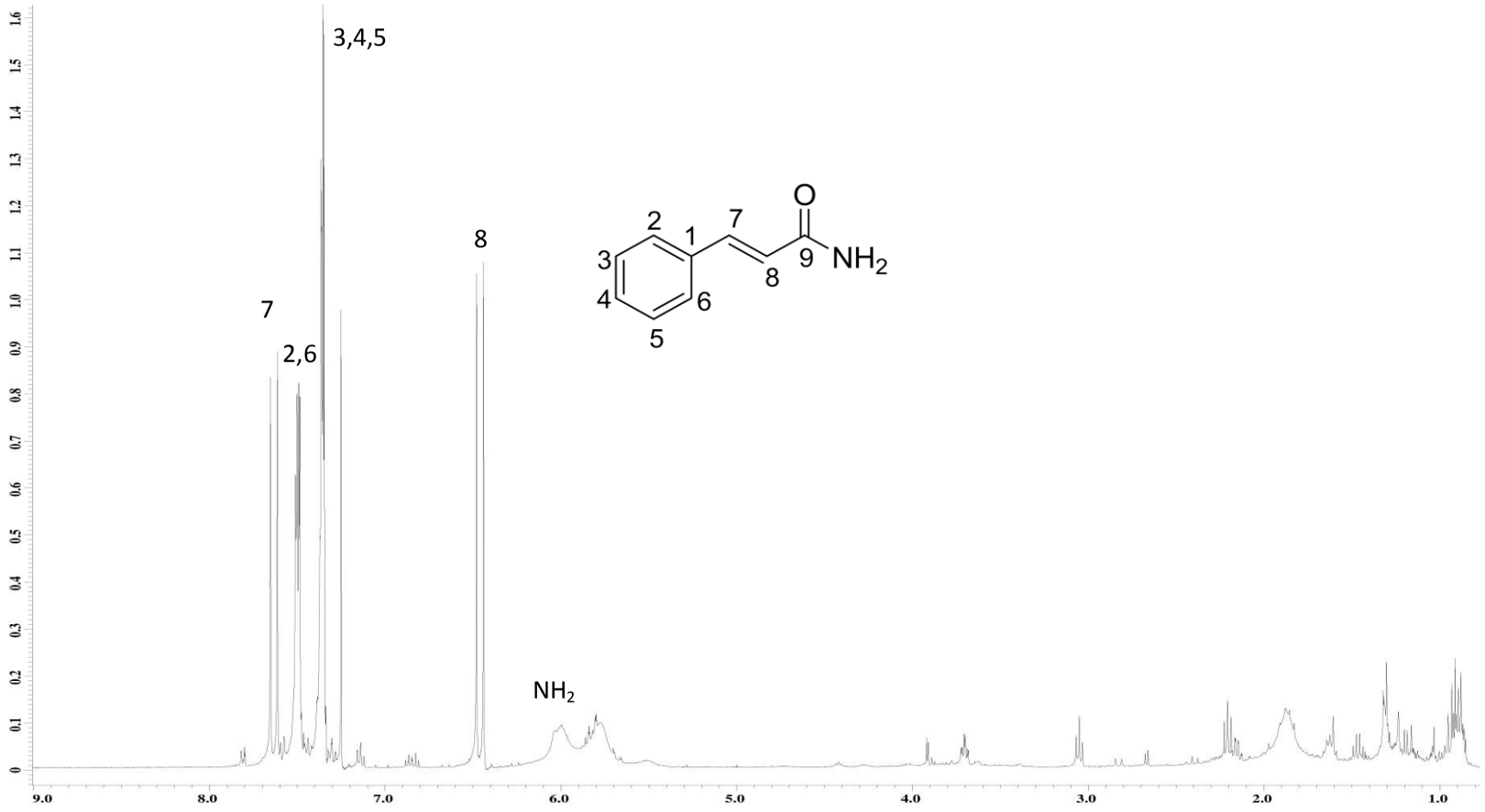


Figure 3.57: ^{1}HNMR Spectrum of Compound **I**

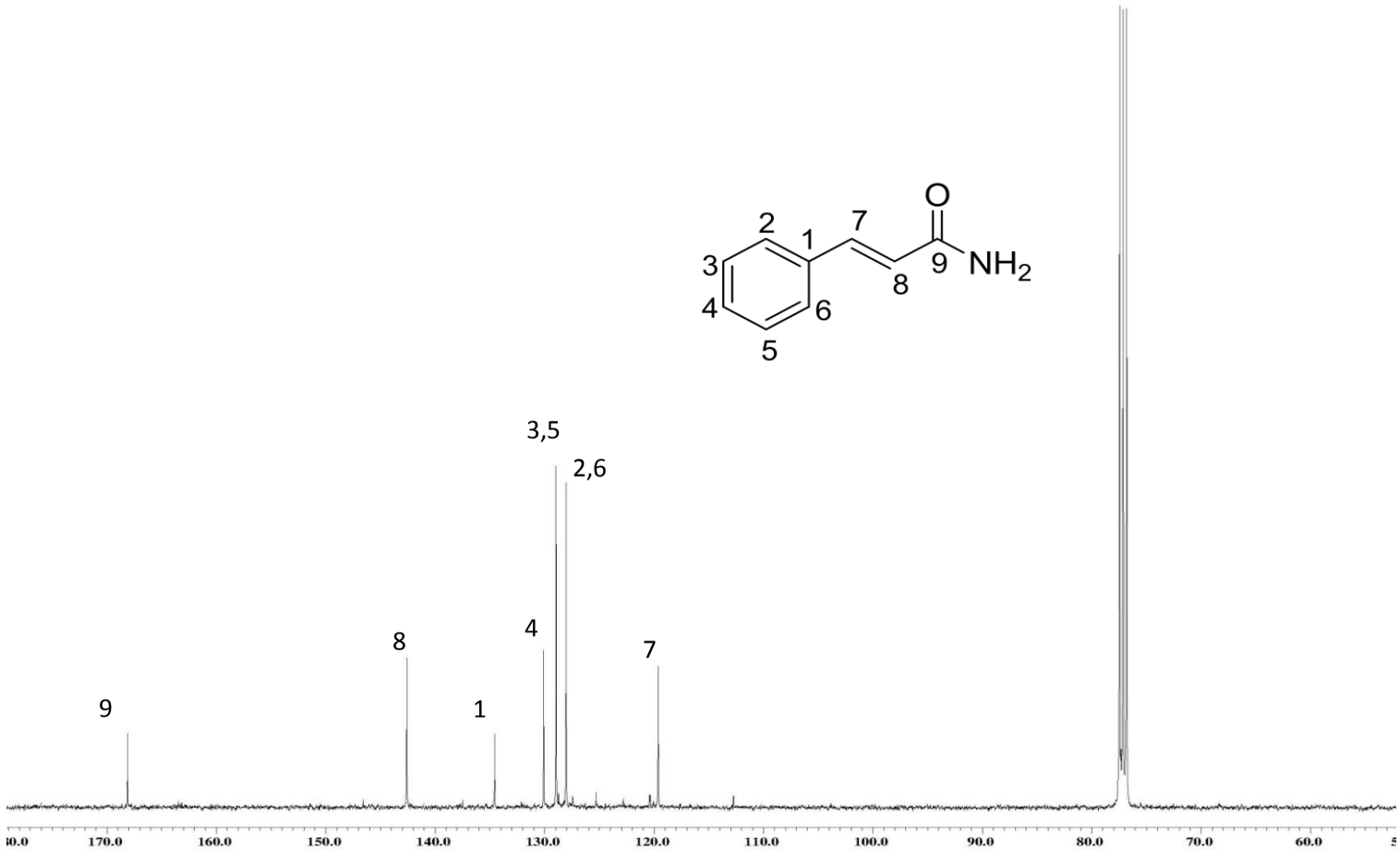


Figure 3.58: ^{13}CNMR Spectrum of Compound **I**

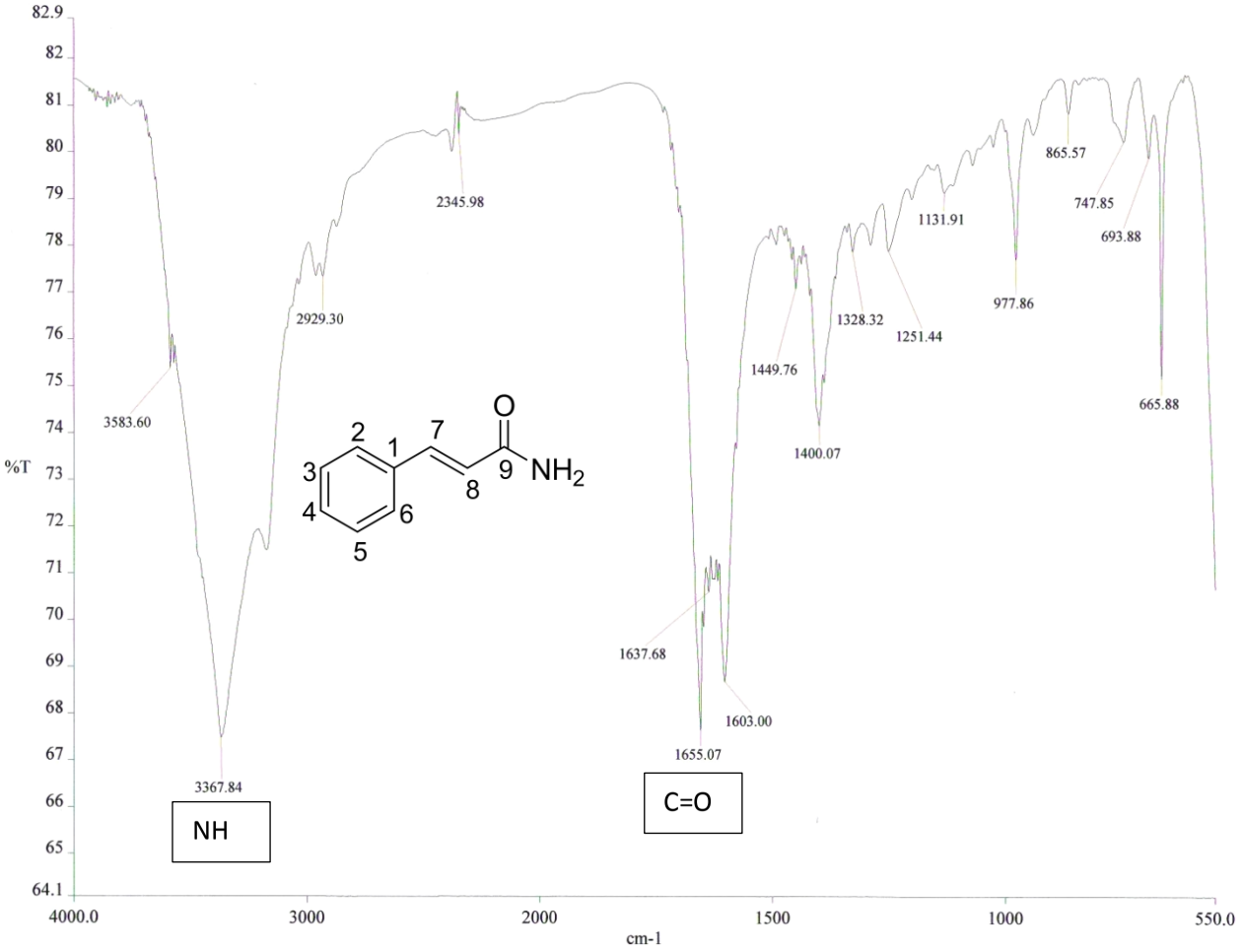


Figure 3.59: IR Spectrum of Compound **I**

3.2 Degree of Unsaturation

All ring and multiple bonds in a compound are called “degree of unsaturation”. One ring or one double bond was counts as one degree of unsaturation, whereas triple bonds count as two degrees of unsaturation. The total degree of unsaturation is calculated from the molecular formula, it can greatly help establish possible structures.

Determining degree of unsaturation from a formula

A general formula for calculating the degrees of unsaturation from a molecular formula is the following:

Degrees of Unsaturation = [(Number of Carbons x 2) + 2 - Number of Hydrogens] / 2

For non-hydrocarbon elements:

Oxygen, O - ignore

Halides (F, Cl, Br, I) - count as a hydrogen

Nitrogen, N - count as one half of a carbon

Example:

Harmane **38**

Molecular Formula $C_{12}H_{10}N_2$

Degree of unsaturation = [(12x2) + 2 – 10 + 2]/2

= 9

CHAPTER 4

VASODILATION ASSAY

VASODILATION ASSAY

4.0 Introduction

Vasodilators are useful for the treatment of cerebral vasospasm and hypertension, and for improvement of peripheral circulation. Several endothelium-dependent vasodilators, such as bradykinin, acetylcholine, and histamine, have been reported to elevate Ca^{2+} levels in endothelial cells and activate NO release, leading to vasorelaxation. On the other hand, contractile response in smooth muscle is caused by an influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels (VDC) and/or receptors operated Ca^{2+}-channels (ROC). The endothelium-independent vasodilators, such as nicardipine, niphedipine, dirtiazem, and verapamil, have been reported to inhibit VDC and led to decrease in the intracellular Ca^{2+} concentration in smooth muscle, leading to vasorelaxation.[71]

4.1 Experimental

Vasodilation Assay

A male Wistar rat weighting 260 g was sacrificed by bleeding from carotid arteries under anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 5 mL KHS

solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3 x 10^{-7} M PE. The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (ACh), and aortic ring in which 80% relaxation occurred, were regarded as tissues with endothelium. When the PE-induced contraction reached a plateau, 10^{-5} M of each sample (**79, 42, 5**) was added.

These animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

4.2 Results and Discussion

When phenylephrine (PE) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response after added neonaucline (**79**; 10^{-5}M), cadamine (**42**; 10^{-5}M), and naucledine (**5**; 10^{-5}M) respectively. The excellent activity could be observed (more than 80% relaxation) after 60 minutes for these three alkaloids after injection of each sample at 1×10^{-5} M as shown in Figure 4. These vasorelaxant effects may be mediated through the increased release of NO from endothelial cells, inhibition of calcium influx from extracellular space through voltage-dependent calcium channels (VDC) and/or receptor-operated Ca^{2+}-channels (ROC), and also through the increased release of NO from endotheliul cells and opening of voltage-gated K^{+}-

channels. Neonaucline **79** is the new compound afforded from *Ochreinauclea maingayii*, while naucledine **5** and cadamine **42** has never been tested on vasorelaxant activity. From this study, cadamine **42** showed the strongest vassodilator with 90% relaxant after 60 minutes, while both neonaucline **79** and naucledine **5** showed about 80% relaxation after 60 minutes.

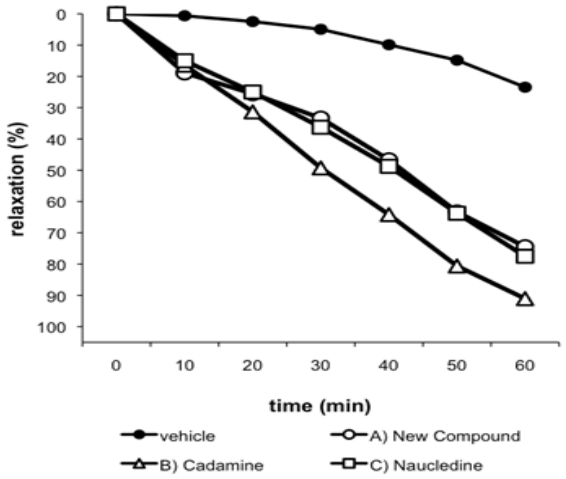


Figure 4.1: Vasorelaxant effects of neonaucline (**79**; 10^{-5}M), cadamine (4**2**; 10^{-5}M), and naucledine (**5**; 10^{-5}M) on endothelium-intact rings cut from rat arteries pre-contracted with PE (0.3 X M

CHAPTER 5

CONCLUSION

CONCLUSION

A phytochemical investigation on the leaves and bark of *Ochreinauclea maingayii* (Hook. f.) Ridsd. with serial number KL5625 (Sg. Tekam, Jerantut, Pahang) and KL5595 (Ulu Sat, Machang, Kelantan) from the family Rubiaceae has been carried out. The isolation and purification of dichloromethane extract produced one new compound; neonaucline **79** along with eight known compounds whereas they were, three indole alkaloids; harmane **38**, cadamine **42**, naucledine **5** and isodihydrocadambine **78**, one *nor*- isoprenoid; blumenol A **75**, two amide; cinnamide **81** and benzamide **80**, and one triterpenoid; ursolic acid **73**. The compounds obtained from the studies are listed in Table 5.1.

Table 5.1: Compounds obtained from *Ochreinauclea maingayii*

	KL5625	KL5595
Leaves	Neonaucline **79** Blumenol A **75** Cadamine **42** Harmane **38** Naucledine **5** Cinamide **81**	Harmane **38** Ursolic acid **73**
Bark	Naucledine **5** Cinnamide **81** Benzamide **80** Isodihydrocadambine **78**	

Vasorelaxation activity study found that the new indole alkaloid, neonaucline **79** showed a significant activity on rat aorta after injection of each sample in 1 x 10^{-5} M when phenylephrine (PE) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response along with the known compound; naucledine **5**, and cadamine **42**.

It can be concurred that indole alkaloids are major compound occuring in *Ochreinauclea maingayii* (Hook. f.) Ridsd. This is the first report on the chemical and biological studies on the genus *Ochreinauclea* and spesies *Ochreinauclea maingayii*. This species can be a source of pharmacologically interesting molecules and more chemical (methanol, ethanol and water extract) and bioactivity studies can be performed such as antioxidant, anticancer or antimalarial on this plant.

CHAPTER 6

EXPERIMENTAL

EXPERIMENTAL

6.1 Instrumentation

- NMR spectra were obtained using JEOL-JNM-LA400 FT NMR Spectrometer System using deuterated chloroform ($CDCl_3$) and deuterated chloroform + deuterated methanol.
- The infrared (IR) spectra were obtained through Perkin Elmer FT-IR Spectrometer Spectrum RX1 using chloroform as solvent.
- Mass spectra were carried out on Shimadzu LCMS-IT-TOFF and GC-MS Spectrometer (HP 6890 Series Mass Selective Detector and HP 6890 Series GC system).
- UV light (245 and 365 nm) were used to examine spots or bands on TLC.

6.2 Chromatography

Column Chromatography

All solvent used in this experiment are industrial graded (distilled). Silica gel 60, were used for column chromatography. Slurry of silica gel (approximate ratio 30:1 silica gel to sample) in hexane system was poured into a glass column of appropriate size with gentle tapping to remove trapped bubbles. The crude extract was initially dissolved in minimum amount and loaded on top of the packed column. The extract was eluted with an appropriate solvent system at a certain flow rate. Fractions of each test

tube were collected and evaporated. Fractions with similar compound were then combining by TLC monitoring.

Thin Layer Chromatography (TLC)

Aluminum supported silica gel plates were used to see the spots of the isolated compounds. UV light was used to examine spots or band on the TLC after spraying with required reagents.

Preparative Thin Layer Chromatography

Plates of size 20 cm x 20 cm were cleaned using soap and rinsed with water, then with acetone and were dried in the oven. The slurry was prepared by adding 60g of silica gel 60 F254 (230-400 Mesh ASTM) or DC-Fertigplatten SIL G-25UV254 to 120 ml distilled water in a closed container, and was shaken vigorously to obtain a smooth mixture. The slurry was then spread onto the clean and dry plates using the Shandon spreading jig with thickness ranging from 0.25 mm to 1 mm, depending on the amount of samples to be worked upon. Then, the plates were activated in the oven for over an hour at 110ºC before use.

6.3 Detector Reagents

Mayer's Reagent (Potassium Mercuric iodide)

The 1.4g of mercuric iodide in 60ml of distilled water mixed with 5.0g of potassium iodide in 10ml of water. The mixture was then made up to 100ml solution. A

positive test result was indicated by the formation of white precipitate when the aqueous layer (acidified) is treated with 2-3 drops of Mayer's reagent.

Dragendorff's Reagent

Solution A: Bismuth (III) nitrate (0.85g) in a mixture of glacial acetic acid (10 ml) and distilled water (40ml).

Solution B: Potassium iodide (8.0g) in distilled water (200 ml).

Stock solution: A mixture of equal volumes of both solution A and B

Spray reagent: The stock solution (20ml) was diluted in a mixture of acetic acid (20ml) and distilled water (60ml).

Vanillin-sulphuric acid vapour

The 0.5 g vanillin in 2 ml concentrated H_2SO_4 was added with cooling to 8 ml ethanol before spraying onto the TLC plate. Dried chromatography TLC plates were sprayed with vanillin reagent. The plates were then heated at 100-105ºC until full development of colors had occurred. The occurrences of blue, red, pink, brown, dark green, grey or purple colours indicated the presence of simple terpene and phenylpropanoid.

6.4 Plant Material

Bark and leaves of *Ochreinauclea maingayii* was collected at Sungai Tekam Reserve Forest, Jerantut, Pahang and Ulu Sat Reserve Forest, Machang, Kelantan with herbarium series number KL5625 and KL5595 respectively. The sample specimen were

identified by Prof. Colin E. Ridsdale (Leiden University, Netherland) and deposited in the herbarium of the Department of Chemistry, University of Malaya.

6.5 Extraction of Plant Materials

Extraction of Leaves of *O. maingayii* KL5625

The 2.0 kg of grounded and dried leaves of the plant were first defatted in 10 L hexane for 3 days at room temperature then filtered and air-dried for 24 hours, and the solvent evaporated to dryness. After being dried, the barks sample was sprinkled with 27% ammonia (NH_3) solution and left to soak overnight. They were then extracted with 10 L dichloromethane CH_2Cl_2 solvent by soaking in a big conical flask for about 3 days. The extract was concentrated to about 500 ml by using the rotary evaporator. This was then followed by re-extraction with 5% hydrochloric acid (HCl) until Mayer's test was negative. The combined extracts were then basified with concentrated ammonia solution to pH 10 -11 and re-extracted with CH_2CI_2. The CH_2Cl_2 extracts were washed with distilled water, followed by sodium chloride solution and finally dried over anhydrous sodium sulphate. The solvents were evaporated to dryness to yield 3.20 g of crude alkaloid. The crude alkaloid fraction was obtained as a dark reddish gummy residue.

Extraction of Bark of *O. maingayii* KL5695

The 1.3 kg grounded and dried bark of *O. maingayii* were first defatted in 10 L hexane for 3 days at room temperature; then filtered and air-dried for 24 hours, and the solvent evaporated to dryness. The dried sample was moistened with 27% ammonia

solution, and then dried it again. It was then extracted with 10 L dichloromethane CH_2Cl_2 solvent by soaked in big conical flask for about 3 days. The solvents were evaporated to dryness to yield 8.70 g of crude CH_2Cl_2 extract. The crude CH_2Cl_2 extract was obtained as a dark brown gummy residue.

Extraction of Leaves of *O.maingayii* KL5595

The same procedure as for the bark of *O. maingayii* KL5695 as described above was used. 1.0 kg of grounded and dried bark of *O.maingayii* KL5595 was first defatted in 10 L hexane for 3 days at room temperature then filtered and air-dried for 24 hours, and the solvent evaporated to dryness. The dried sample was moistened with 27% ammonia solution, and then dried it again. It was then extracted with 10 L dichloromethane ($CH_2Cl_{2)}$ solvent by soaking in a big conical flask for about 3 days. The solvent was evaporated to dryness to yield 8.70 g of crude CH_2Cl_2 extract. The crude CH_2Cl_2 extract was obtained as a dark green residue.

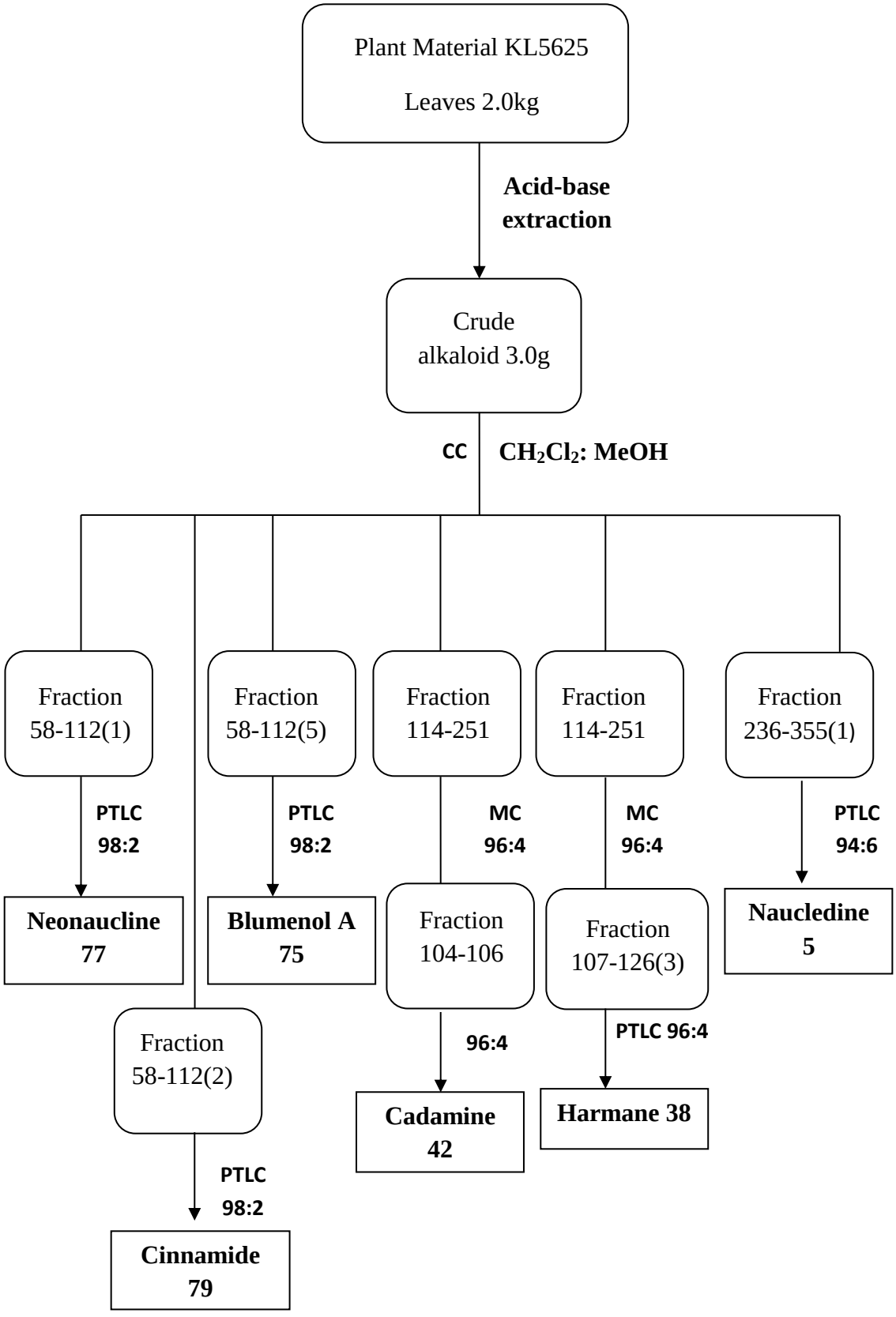
6.6 Separation and Purification

The basic work up on the dichloromethane crude extract of the plant follow the same general procedure described below. The crude fraction was subjected to column chromatography over silica gel, using the g solvent system as stated at the Table 6.1.

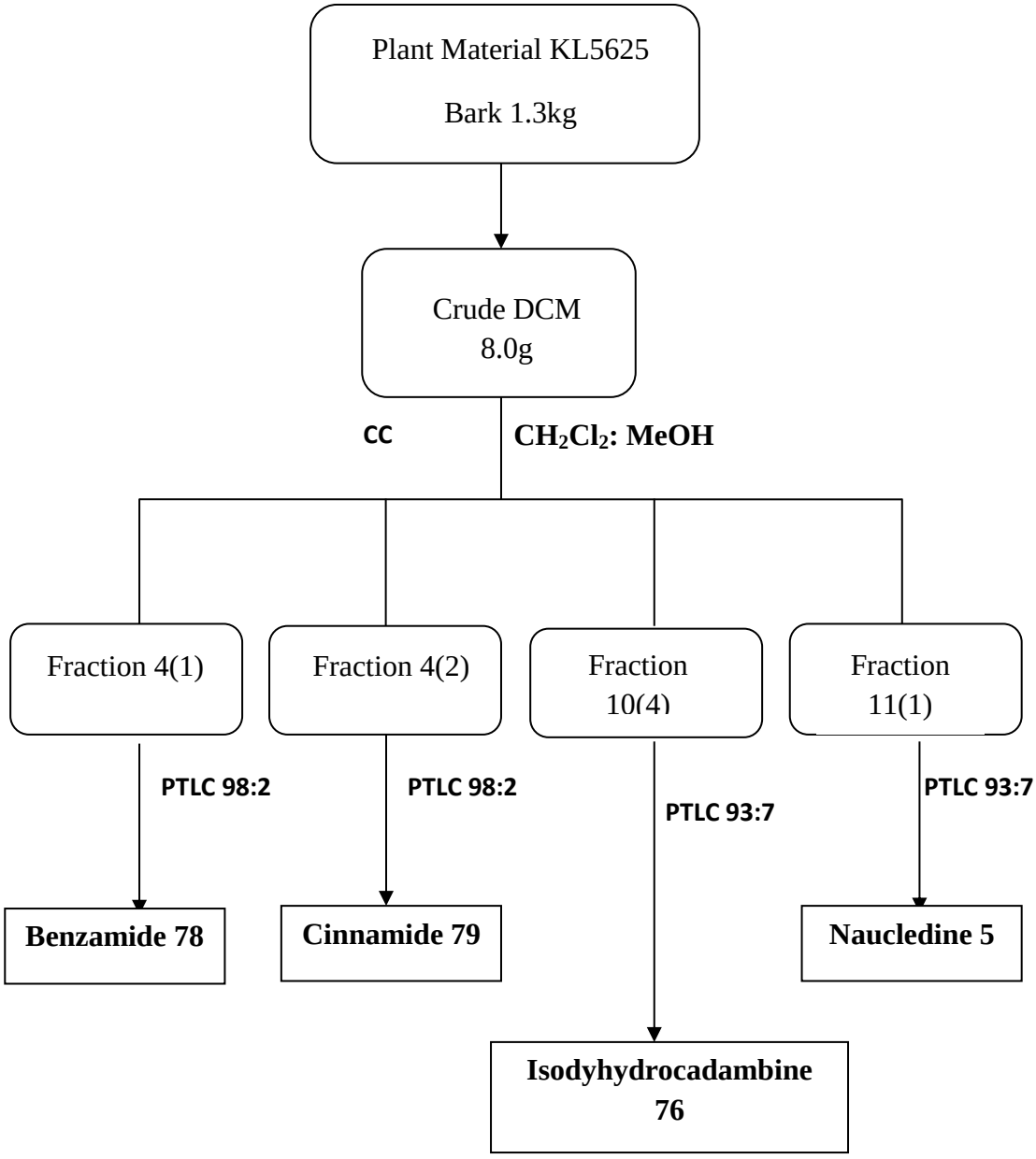
Table 6.1: Solvent system Dichloromethane: Methanol

Dichloromethane	Methanol
100	0
98	2
95	5
85	15
80	20
75	25
70	30
60	40
50	50
30	70
0	100

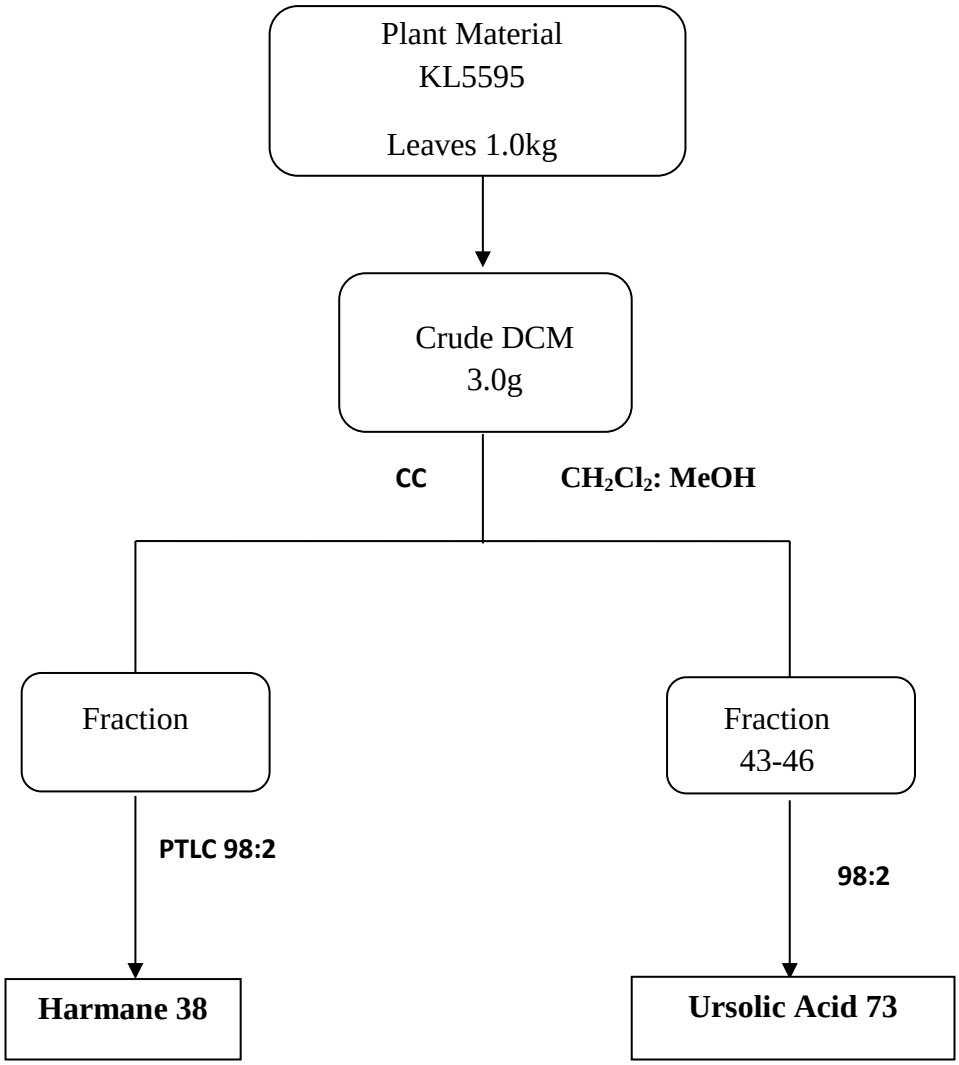
The fractions were collected and grouped into a series of fractions and monitored by thin layer chromatography (TLC). Each series was than treated separately to isolate and purify its chemical constituent contents either by extensive column chromatography or by preparative TLC. The summarized of the isolation and purification of obtained compounds were displayed at the Scheme 6.1, 6.2, and 6.3.



Scheme 6.1: Isolation and Purification of Compounds from The Leaves of *Ochreinauclea maingayii* (KL5695)



Scheme 6.2: Isolation and Purification of Compounds from The Bark of *Ochreinauclea maingayii* (KL5695)



Scheme 6.3: Isolation and Purification of Compounds from The Leaves of *Ochreinauclea maingayii* (KL5595)

6.7 PHYSICAL AND SPECTRAL DATA OF ISOLATED COMPOUNDS

Harmane 38 **: $C_{12}H_{10}N_2$**

UV λ_{max} nm : 347, 334, 287, 239, 234, and 212

IR V_{max} cm^{-1} : 3125

Mass Spectrum *m/z* $[M+H]^+$: 183.0921

^1H NMR ($CDCl_3$) δ ppm : H-5 (8.33 *d J*=5.4), H-6 (7.80 *d J*=5.4), H-9 (8.09 *d J*=7.8), H-10 (7.26-7.23 *m)*, H-11 (7.52-7.50 *m*), H-12 (7.52-7.50 *m*), H-14 (2.81 *s*)

^{13}C NMR ($CDCl_3$) δ ppm : C-2 (142.0), C-3 (134.5), C-5 (138.0), C-6 (112.6), C-7 (128.2), C-8 (122.2), C-9 (122.0), C-10 (120.0), C-11 (111.4), C-12 (128.0), C-13 (140.5), C-14 (21.0)

Naucledine 5 **: $C_{18}H_{15}N_3O_2$**

UV λ_{max} nm : 330 and 218

IR V_{max} cm^{-1} : 3402

Mass Spectrum *m/z* $[M+H]^+$: 306.1213

^1H NMR ($CDCl_3$) δ ppm : H-5 (4.13 *t J*= 6.8), H-6 (3.03 *t J*= 6.8), H-9 (7.68 *d J*= 8.3), H-10 (7.23 *dd J*=7.8, 8.3), H-11 (7.33 *dd J*=7.8, 8.3), H-12 (7.42 *d J*= 8.3), H-15 (9.30 *d J*=2.0), H-16(9.18 *d J*=2.0), H-19 (8.68 *t J*=2.0)

^{13}C NMR ($CDCl_3$) δ ppm : C-2 (126.3), C-3 (156.3), C-5 (49.3), C-6 (19.3), C-8 (125.4), C-9 (120.3), C-10 (120.9), C-11 (125.5), C-12 (112.4), C-13 (136.9), C-14 (125.4), C-15 (151.8), C-17 (152.6), C-18 (133.3), C-19 (136.5), C-20 (165.5), C-21 (52.8)

Cadamine 42 **: $C_{21}H_{21}N_3O_3$**

UV λ_{max} nm : 324 and 222

IR V_{max} cm^{-1} : 3306.64

Mass Spectrum *m/z* $[M+H]^+$: 364.1638

^1H NMR ($CDCl_3$) δ ppm : H-3 (4.43 *m*), H-5 (2.94 *m*), H-5'(3.22 *m*), H-6 (2.80 *m*), H-6' (2.98 *m*), H-9 (7.48 *d J*=8.0), H-10 (7.10 *dd J*=8.0, 8.0), H-11 (7.16 *dd J*=8.0, 8.0), H-12 (7.27 *d J*=8.0), H-14 (3.25 *m*), H-14' (3.51 *m),* H-17 (8.51 *s*), H-19 (8.96 *s*), H-22 (3.88 *s*), H-23 (4.10 *m*), H-24 (3.61 *m*), H-24' (3.75 *m*)

^{13}C NMR ($CDCl_3$) δ ppm : C-2 (134.9), C-3 (46.3), C-5 (47.6), C-7 (107.9), C-8 (126.9), C-9 (118.3), C-10 (119.4), C-11 (121.9), C-12 (111.1), C-13 (136.2), C-14 (28.3), C-15 (124.5), C-16 (130.8), C-17 (151.8), C-19 (150.0), C-20 (144.8), C-21 (166.3), C-22 (52.4), C-23 (62.6), C-24 (63.6)

Isodyhydrocadambine 78 **: $C_{27}H_{34}N_2O_{10}$**

UV λ_{max} nm : 275, 271 and 220

IR V_{max} cm^{-1} : 3369 and 1692

Mass Spectrum *m/z* $[M+H]^+$: 547.2335

^1H NMR

($CDCl_3$ + CD_3OD) δ : H-3 (3.70-3.74 *m*), H-5a (2.95-2.99 *m*), H-5b (3.09-3.14 *m),* H-6a (2.68 *m*), H-6b (2.82-2.99 *m*), H-9 (7.38 *d J*=7.8), H-10 (7.01 *t J*=7.3), H-11 (7.07 *t J*=7.3*),* H-12 (7.25 *d J*=8.2), H-14a (1.64-1.73 *m*), H-14b (2.16 *d J*=14.2), H-15 (2.95-2.99 *m*), H-17 (7.47 *s*), H-18a (2.87-

2.89 *m)*, H-18b (3.09-3.14 *m*), H-19 (4.24 *t J*=5.9), H-20 (1.87-1.92 *m*), H-21 (5.38 *d J*=9.1), H-1’ (4.75 *d J*=7.8), H-2’ (3.36-3.40 *m*), H-3’ (3.44-3.51 *m*), H-4’ (3.44-3.51 *m)*, H-5’ (3.20-3.23 *m*), H-6’ (3.69-3.72 *m*), H-23 (3.73 *s*)

^{13}C NMR

($CDCl_3$ + CD_3OD) δ : C-2 (134.4), C-3 (63.4), C-5 (55.0), C-6 (22.7), C-7 (108.2), C-8 (126.8), C-9 (118.0), C-10 (119.3), C-11 (121.6), C-12 (111.1), C-13 (136.4), C-14 (36.5), C-15 (33.1), C-16 (109.7), C-17 (153.2), C-18 (58.3), C-19 (64.5), C-20 (43.1), C-21 (96.2), C-22 (168.6), C-1’ (99.8), C-2’ (72.7), C-3’ (76.5), C-4’ (68.9), C-5’ (76.8), C-6’ (60.8), C-23 (51.7).

Neonaucline 79 **: $C_{20}H_{15}N_3O_3$**

UV λ_{max} nm : 376, 284 and 204

IR V_{max} cm^{-1} : 3364 and 1731

Mass Spectrum *m/z* $[M+H]^+$: 346.1140

^{1}H NMR ($CDCl_3$) δ : H-5 (4.54 *t J*=6.8), H-6 (3.19 *t J*=6.8), H-9 (7.64 *d J*=7.8), H-10 (7.18 *dd J*=7.8, 7.8), H-11 (7.35 *dd J*=7.8, 7.8), H-12 (7.46 *d J*=7.8), H-14 (7.88 *s*), H-17 (9.32 *s*), H-19 (9.69 *s*), H-22 (4.00 *s*), N-H (8.72 *bs*)

^{13}C NMR ($CDCl_3$) δ : C-2 (127.4), C-3 (138.2), C-5 (40.7), C-6 (19.4), C-7 (116.9), C-8 (125.7), C-9 (119.9), C-10 (120.9), C-11 (125.6), C-12 (111.9), C-13 (138.6), C-14 (95.1), C-15 (141.9), C-16 (117.8), C-17 (154.2), C-19 (155.4), C-20 (120.4), C-21 (166.4), C-22 (52.5), C-23 (166.4)

Ursolic Acid 73 **: $C_{30}H_{48}O_3$**

UV λ_{max} nm : 472, 444, and 421

IR V_{max} cm^{-1} : 3421

Mass Spectrum *m/z* $[M+H]^+$: 457.2800

^1H NMR

($CDCl_3$ + CD_3OD) δ : H-1 (0.89-0.86 *m*), H-2 (1.08-1.02 *m*), H-3 (3.14 *dd J*=9.6, 6.4), H-5 (0.68 *d J*=10), H-6 (1.49-1.45 *m*), H-7 (1.39-1.26 *m*), H-9 (1.49-1.44 *m*), H-11 (1.85-1.81 *m*), H-12 (5.18 *m*), H-15 (1.64-1.57 *m*), H-16 (1.60-1.53 *m*), H-18 (2.15 *d J*=11.0), H-19 (1.30-1.28 *m*), H-20 (0.97-0.92 *m*), H-21 (1.44-1.42 *m*), H-22 (1.67-1.60 *m*), H-23 (0.92 *s*), H-24 (0.72 *s*), H-25 (0.86 *s*), H-26 (0.74 *s*), H-27 (1.08 *s*), H-29 (0.81 *d J*= 6.0), H-30 (0.89 *d J*= 6.0)

^{13}C NMR

($CDCl_3$ + CD_3OD) δ : C-1 (38.6), C-2 (28.1), C-3 (78.9), C-4 (39.5), C-5 (55.3), C-6 (18.3), C-7 (33.1), C-8 (39.1), C-9 (47.8), C-10 (36.8), C-11 (23.3), C-12 (125.6), C-13 (138.2), C-14 (42.1), C-15 (26.9), C-16 (24.2), C-17 (47.6), C-18 (52.8), C-19 (38.9), C-20 (38.7), C-21 (30.7), C-22 (36.9), C-23 (28.1), C-24 (15.6), C-25 (15.5), C-26 (17.0), C-27 (23.6), C-28 (180.8), C-29 (16.9), C-30 (21.2)

Blumenol A 75 **: $C_{13}H_{20}O_2$**

UV λ_{max} nm : 237

IR V_{max} cm^{-1} : 3413, 2968, 1651 and 1372

Mass Spectrum *m/z* $[M+H]^+$: 247.1334

^{1}H NMR ($CDCl_3$) δ : H-2β (2.24 *d J*=17.1), H-2α (2.45 *d J*=17.1), H-4 (5.90 *s*), H-7 (5.79 *d J*=15.7), H-8 (5.85 *d J*=15.7), H-9 (4.41*m*), H-10 (1.30 *d J*=6.3), H-11 (1.02 *s*), H-12 (1.08 *s*), H-13 (1.90 *s*)

^{13}C NMR ($CDCl_3$) δ : C-1 (41.1), C-2 (49.7), C-3 (198.0), C-4 (126.9), C-5 (162.7), C-6 (79.0), C-7 (135.8), C-8 (129.0), C-9 (98.0), C-10 (23.8), C-11 (22.9), C-12 (24.0), C-13 (18.9)

Benzamide 80 : C_7H_7O

Melting Point °C : 125-127

UV λ_{max} nm : 294 and 280

IR V_{max} cm^{-1} : 3365 cm^{-1}

Mass Spectrum *m/z* $[M+H]^+$: 121

^{1}H NMR ($CDCl_3$) δ : H-3 (7.78 *d J*=7.3), H-4 (7.42 *t J*=7.3), H-5 (7.49 *t J*=7.3), H-6 (7.42 *t J*=7.3), H-7 (7.78 *d J*=7.3).

^{13}C NMR ($CDCl_3$) δ : C-1 (169.4), C-2 (133.4), C-3 (127.3), C-4 (128.6), C-5 (131.9), C-6 (128.6), C-7 (127.3)

Cinnamide 81 : C_9H_9NO

Melting Point °C : 133-135

UV λ_{max} nm : 217 and 272

IR V_{max} cm^{-1} : 3368 and 1655

Mass Spectrum *m/z* $[M+H]^+$: 146.00

^{1}H NMR ($CDCl_3$) δ : H-2 (7.36 *m*), H-3 (7.50 *m*), H-4 (7.50 *m*), H-5 (7.50 *m*), H-6 (7.36 *m*), H-7 (7.46 *d J*=16.0), H-8 (7.63 *d J*=16.0), NH_2 (5.95 *bs*)

^{13}C NMR ($CDCl_3$) δ : C-1 (134.6), C-2 (128.0), C-3 (128.9), C-4 (130.1), C-5 (128.9), C-6 (128.0), C-7 (142.6), C-8 (119.6), C-9 (168.1)

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APPENDICES

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Article

Neonaucline, a New Indole Alkaloid from the Leaves of *Ochreinauclea maingayii* (Hook. f.) Ridsd. (Rubiaceae)

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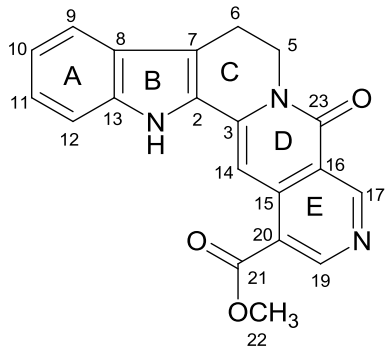
Abstract: A new indole alkaloid; neonaucline (**1**), along with six known compounds–Cadamine (**2**), naucledine (**3**), harmane, benzamide, cinnamide and blumenol A–were isolated from the leaves of *Ochreinauclea maingayii* (Rubiaceae). In addition to that of compound **1**, ^{13}C-NMR data of cadamine (**2**) and naucledine (**3**) were also reported. Structural elucidations of these alkaloids were performed using spectroscopic methods especially 1D- and 2D-NMR, IR, UV and LCMS-IT-TOF. The excellent vasorelaxant activity on isolated rat aorta was observed for the alkaloids **1**–**3** after injection of each sample at 1×10^{-5} M.

Keywords: *Ochreinauclea maingayii*; neonaucline; nauledine; cadamine; Rubiaceae; alkaloid

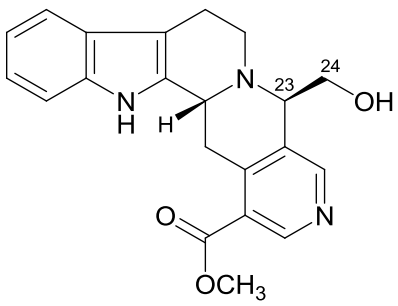
1. Introduction

Ochreinauclea maingayii (Hook. f.) Ridsd. (Rubiaceae) is a medium size tree distributed in Peninsular Malaysia, Borneo, Sumatra and Thailand [1-4]. The timber of *Ochreinauclea* species shares the standard Malaysian name "*bangkal*" or "mengkal" with *Nauclea* and *Neonauclea* species [5]. There has been no report of other phytochemical study and medicinal value of *Ochreinauclea maingayii* so far. In continuation of our research on plants from the Rubiaceae family [6], we have embarked a study on the CH_2Cl_2 extract of the plant *Ochreinauclea maingayii*. The present study has led to the isolation of a new indole alkaloid, neonaucline (**1**) together with cadamine (**2**) [7-9], naucledine (**3**) [10-13], harmane [14-15], blumenol A [16], bezamide, and cinnamide.

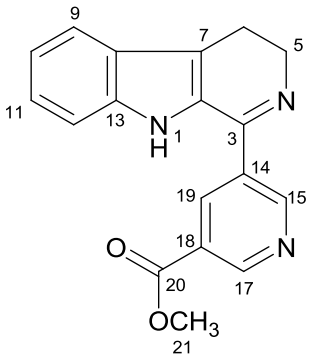
Figure 1. Structures of neonaucline (**1**), cadamine (**2**), and naucledine (**3**).



(**1**)



(**2**)



(**3**)

2. Results and Discussion

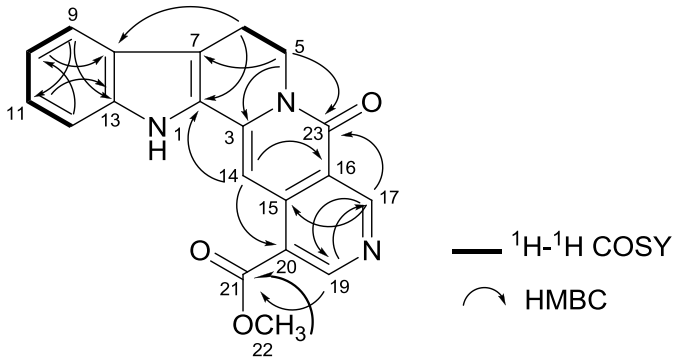
Neonaucline (**1**) was isolated as a yellowish amorphous solid. The LCMS-IT-TOF spectrum of **1** showed a pseudomolecular ion peak, [M+H] at *m/z* 346.1140, corresponding to the molecular formula of $C_{20}H_{16}N_3O_3$. The IR spectrum revealed absorption bands at 3,364 and 1,731 cm^{-1} for the stretching vibrations of –NH and –CO groups respectively. In the ^{1}H-NMR spectrum, signals for seven aromatic protons due to one methoxy singlet and one $–CH_2–CH_2–N–$ group were observed, thus suggesting an indolopyridinequinolizinone type of skeleton [17]. Among the seven aromatic proton signals, two resonated as doublet of doublets (dd) at δ 7.35 and 7.18 (H-10 and H11), two doublets at δ 7.64 and 7.46 (H-9 and H-12), and three singlets at δ 7.88, 9.32 and 9.69 assignable to H-14, H-17, and H-19, respectively. Further analysis of the ^{1}H-NMR and ^{13}C-NMR spectra showed that **1** is very similar to naucletine [18] except that the former revealed the presence of a singlet representing a methoxy group at δ_H 4.00 and

δ_C 52.5. The position of COOMe attached to C-20 (δ 120.4) in ring E was confirmed based on the HMBC correlations of H-14/C-20 (δ 120.4), H-19/C-21 (δ 166.4), H-22/C-21 and H-17/C-23 (δ 166.4), respectively as shown in Figure 2. The ^{13}C-NMR spectrum revealed 20 carbon signals due to eight quaternary carbons, seven methines, two methylenes, one methoxy group and two carbonyl groups. The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectral assignments performed by extensive 2D-NMR experiments (COSY, HSQC and HMBC) were summarized in Figure 2 and Table 1.

Table 1. ^{1}H-NMR (400 Hz) and ^{13}C-NMR (100 Hz) spectral data of neonaucline (**1**) and cadamine (**2**) in $CDCl_3$.

Position	**^{1}H (δ_H, Hz) (1)**	**^{13}C (δ_C, $CDCl_3$)**	**^{1}H (δ_H, Hz) (2)**	**^{13}C(δ_C,$CDCl_3$)**
N-H	8.72s	-	7.95s	-
2	-	127.4	-	134.9
3	-	138.2	4.43m	46.3
5	4.54t (2H,6.8)	40.7	2.94, 3.22m	47.6
6	3.19t (2H,6.8)	19.4	2.80, 2.98m	21.9
7	-	116.9	-	107.9
8	-	125.7	-	126.9
9	7.64d (7.8)	119.9	7.48 d (8.0)	118.3
10	7.18dd (7.8,7.8)	120.9	7.10 dd (8.0,8.0)	119.4
11	7.35dd (7.8,7.8)	125.6	7.16 dd (8.0,8.0)	121.9
12	7.46d (7.8)	111.9	7.27 d (8.0)	111.1
13	-	138.6	-	136.2
14	7.88 s	95.1	3.25,3.51 m	28.3
15	-	141.9	-	124.5
16	-	117.8	-	130.8
17	9.32 s	155.4	8.51 s	151.8
19	9.69 s	154.2	8.96 s	150.0
20		120.4	-	144.8
21	-	166.4	-	166.3
22-OMe	4.00 s	52.5	3.88 s	52.4
23	-	166.4	4.10 m	62.6
24	-	-	3.61, 3.75 m	63.6

Figure 2. Selected 2D NMR correlations of neonaucline (**1**).



Cadamine (**2**) and naucledine (**3**) were isolated as a reddish and yellowish amorphous solid. The LCMS-IT-TOFF spectra showed pseudomolecular ion peaks, $[M+H]^+$ at *m/z* 364.1638 [$C_{21}H_{21}N_3O_3$] and *m/z* 306.1213 [$C_{18}H_{15}N_3O_3$], respectively. The ^{1}H-NMR data of cadamine (**2**) was reported previously based on cadamine acetate [7] whereas **3** were first isolated from *Nauclea diderrichi* [10]. We herein report the ^{13}C-NMR data for both compounds which has not been reported yet [7-13]. In view of that, complete assignments were established through various NMR measurements; DEPT, HMQC, HMBC and NOESY spectra. The ^{13}C-NMR spectra of cadamine (**2**) and naucledine (**3**) indicated the presence of 21 and 18 carbons, respectively, as shown in Tables 1 and 2.

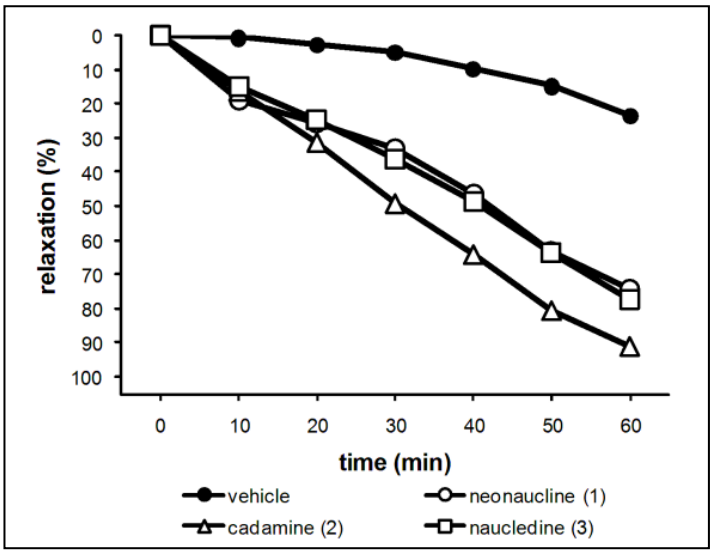
Table 2. ^{1}H-NMR (in DMSO-d6 and $CDCl_3$) and ^{13}C-NMR (100 Hz, $CDCl_3$) spectral data of naucledine (**3**).

Position	***^{1}H (τ, DMSO-d_6)**	**^{1}H (δ_H, Hz)**	**^{13}C (δ_C,)**
N-H	0.90s	8.35 s	
2	-	-	126.3
3	-	-	156.3
5	6.36m	4.13 (t, 6.8)	49.3
6	6.96m	3.03 (t, 6.8)	19.3
7	-	-	
8	-	-	125.5
9		7.68 (d, 8.3)	120.3
10	2.2-3.1	7.23 (dd, 7.8, 8.3)	120.9
11	(3peaks)	7.33 (dd, 7.8,8.3)	125.4
12		7.42 (d, 8.3)	112.4
13	-	-	136.9
14	-	-	125.5
15	0.65d	9.30 (d, 1.96)	151.8
17	0.73d	9.18 (d, 1.96)	152.6
18	-	-	133.3
19	0.88t	8.68 (t, 1.96)	136.5
20			165.5
21	6.08s(3H)	3.98 (s)	52.8

* ^{1}H-NMR data is reproduced from Murray *et al*. [11].

Vasodilators are useful for treatment of cerebral vasospasm and hypertension, and for improvement of peripheral circulation [19]. When phenylephrine (PE) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response, we added neonaucline (**1**; 1×10^{-5}M), cadamine (**2**; 1×10^{-5}M), and naucledine (**3**; 1×10^{-5}M). The excellent activity could be observed for these three alkaloids (**1**–**3**) after injection of each sample at 1×10^{-5}M as shown in Figure 3.

Figure 3. Vasorelaxant effects of neonaucline (**1**; 10^{-5}M), cadamine (**2**; 10^{-5}M), and naucledine (**3**; 10^{-5}M) on endothelium-intact rings cut from rat arteries pre-contracted with PE (0.3 μM).



In the previous papers, we have reported vasorelaxant activities of some bisbenzylisoquinoline alkaloids such as α′-oxoperakensimines A–C from *Alseodaphne perakensis* and *A. corneri* [20,21], and *N*-allyllaurolitsine from *Litsea lancifolia* [22]. These vasorelaxant effects may be mediated through the increased release of NO from endothelial cells, inhibition of calcium influx from extracellular space through voltage-dependent calcium channels (VDC) and/or receptor-operated Ca^{2+}-channels (ROC), and also through the increased release of NO from endotheliul cells and opening of voltage-gated
K^{+}-channels. The mode of action of these alkaloids on vasorelaxant activity is under investigation.

3. Experimental

General

Spectra were recorded on the following instruments: UV, Shimadzu UV-160A UV-Visible spectrophotometer; IR, Perkin Elmer 1600; NMR, JEOL ECA 400 MHz; LCMS-IT-TOF, Shimadzu. All solvents, except those used for bulk extraction are AR grade. Silica gel 60 F_{254} was used for column chromatography. Glass and aluminium supported silica gel 60 F_{254} plates were used for preparative TLC. TLC sports were visualized under UV light (254 and 365 nm) followed by spraying with Dragendorff's reagent for alkaloid detection.

Plant Material: The leaves of *Ochreinauclea maingayii* were collected at Reserve Forest Sg. Tekam, Jerantut, Pahang, Malaysia, in 17 February 2009. The plant species was identified by Prof. Colin E. Ridsdale from Leiden University, Netherland. A voucher specimen (KL5625) was deposited in the Herbarium of Department of Chemistry, University of Malaya, Malaysia.

Extraction and the isolation: A total of 2.0 kg of dried and grounded leaves of *Ochreinauclea maingayii* was extracted with CH_2Cl_2. Extraction of alkaloids was carried out in the usual manner, which has been described in detail [17-19]. Finally, the extract was concentrated to give crude alkaloids of 3.0 g in weight. The isolation and

purification of compounds **1**–**3** by a small column chromatography (column dimension = 1.0 cm, length = 25 cm, silica gel 60, 70–230 mesh ASTM; Merck 7734) and preparative TLC (PTLC Merck KGaA silica gel 60 F_{254}) yielded 0.13% of neonaucline (**1**), (CH_2Cl_2-MeOH; 98:2), cadamine (**2**), (0.76%, CH_2Cl_2-MeOH; 96:4) and naucledine (**3**), (0.08%, CH_2Cl_2-MeOH; 96:4), respectively.

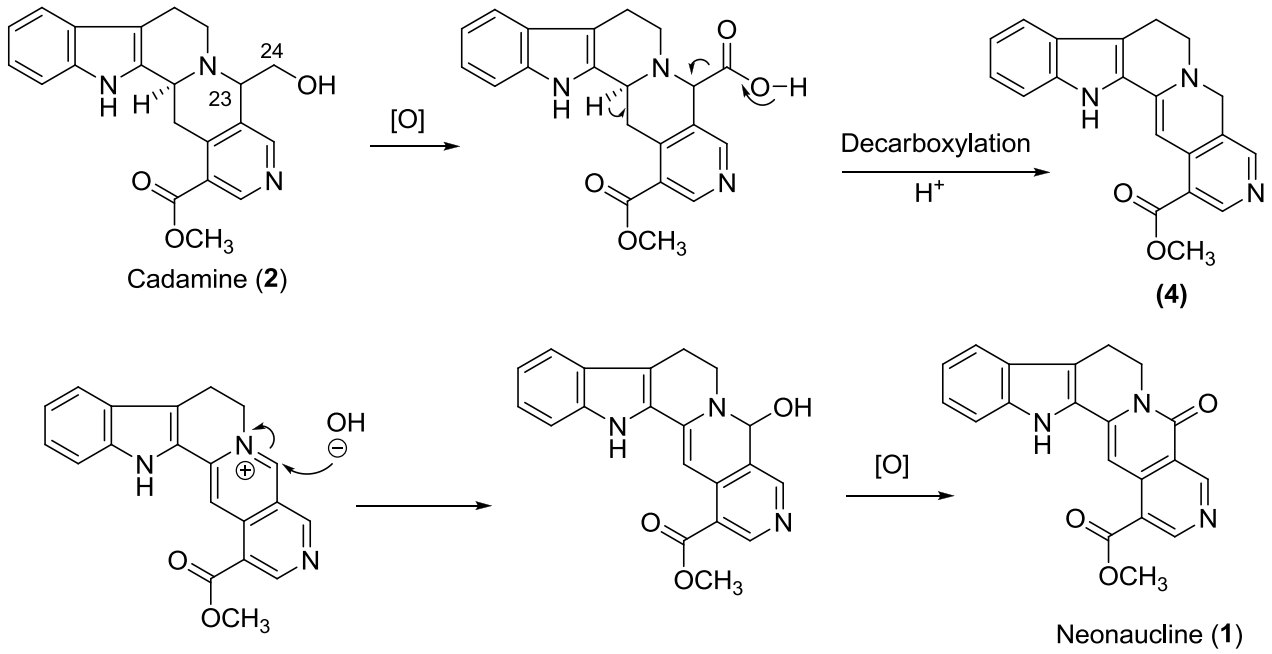
Vasodilation Assay: The vasorelaxant activities of all the alkaloids **1**–**3** were tested using the same procedure as reported previously by Morita *et al.* [20]. The animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

Neonaucline (**1**). Yellowish amorphous solid, LCMS-IT-TOFF at *m/z* 346.1140 ($[M+H]^+$; calcd for $C_{20}H_{16}N_3O_3$, 346.1192); UV (MeOH) 376, 284, 204nm; IR (KBr) λ_{max} 3,364, 1,737 and 1,731 cm^{-1}; ^1H and ^{13}C-NMR: see Table 1.

4. Conclusions

This is the first report on the phytochemical and biological studies on the species of *Ochreinauclea maingayii*. One new indole alkaloid- neonaucline (**1**) along with six known compounds- three alkaloids (cadamine **2**, naucledine **3** and harmane), one nor-isoprenoid-type (blumenol A), bezamide and cinnamide were isolated from the leaves of this species. The proposed biogenesis of **1** from cadamine (**2**) is illustrated in Scheme 1. The latter is oxidized at C-24 followed by decarboxilation and protonation of the carbanion to give compound **4**. Then, **4** will be hydroxylated at C-23 followed by oxidation to give neonaucline (**1**). The excellent vasorelaxant activity on isolated rat aorta was observed for the alkaloids **1**–**3** after injection of each sample at 1×10^{-5} M.

Scheme 1. Biogenetic pathway for neonaucline (**1**).



Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Sample Availability: Not available.